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(54) Title: MONOCLONAL ANTIBODIES FOR INDUCING TOLERANCE (57) Abstract Tolerance to an antigen is induced in a subject by administering a non-depleting CD4 monoclonal antibody and a non-depleting CD8 monoclonal antibody. Tolerance to the antigen can be induced under cover of these antibodies. A depleting CD4 monoclonal antibody and/or a depleting CD8 monoclonal antibody may be administered prior to the non-depleting antibodies.		

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MONOCLONAL ANTIBODIES FOR INDUCING TOLERANCE

This invention relates to tolerance induction using monoclonal antibodies.

5 Tolerance to foreign antigen or tissue, or self antigen or tissue is a state whereby an otherwise normal, mature immune system is specifically unable to respond aggressively to that antigen/tissue which it therefore treats like a normal (non-diseased) body
10 tissue/component, yet at the same time it can respond aggressively to foreign or diseased antigens/tissues to which it has not been specifically made tolerant by the natural process of self tolerance or by therapeutic tolerance induction procedures. A test for tolerance
15 usually requires a demonstration that the tolerant individual fails to become immune to the specific antigen/tissue when one or preferably more attempts to immunize are made at a later time when the same individual can be shown to respond to an irrelevant
20 antigen/tissue.

Monoclonal antibodies (mAb) against the murine CD4 (L3T4) antigen have proven to be potent immunosuppressive agents for the control of humoral immunity, transplant rejection and autoimmunity. In
25 addition, CD4 mAbs have been shown to create a tolerance-permissive environment in vivo with which can be achieved tolerance to certain soluble protein antigens as well as transplantation antigens. However, the mechanism(s) by which CD4 mAbs produce these effects are still not clear.
30 In most previous reports, immunosuppression was obtained under conditions that depleted target cells in vivo. A simple interpretation was that the immune suppression so achieved was due to the absence of CD4 T cells.

On the other hand, in vitro work has demonstrated
35 that CD4 (and CD8) mAbs could affect lymphocyte functions

simply through binding to the antigen on the cell surface without cell lysis. In addition, immunosuppression and tolerance induction has been obtained in vivo with the use of sublytic concentrations of CD4 mAbs and by F(ab')₂ CD4 mAb fragments which suggest that for mAb-mediated immune regulation, depletion of target cells may not be essential.

Previous studies have used antibodies that deplete CD4 cells. We have now found that non-depleting CD4 and CD8 antibodies can also produce tolerance to foreign immunoglobulins, bone marrow and skin grafts. Indeed, this observation has general applicability to all antigens. Further, we have found that administration of a depleting CD4 mAb and/or a depleting CD8 mAb prior to administration of the non-depleting mAbs can be beneficial in creating a tolerance-permissive environment.

The present invention therefore provides products containing a non-depleting CD4 mAb and a non-depleting CD8 mAb as a combined preparation for simultaneous, separate or sequential use in inducing tolerance to an antigen. A non-depleting CD4 mAb for use in a method of treatment of the human or animal body by surgery or therapy and a non-depleting CD8 mAb for use in a method of treatment of the human or animal body by surgery or therapy also form part of the invention. The invention further provides:

- non-depleting CD4 and CD8 mAbs for use together in a method of treatment of the human or animal body by surgery or therapy, especially for use in inducing tolerance to an antigen; and
- a pack comprising as separate components a non-depleting CD4 mAb and a non-depleting CD8 mAb, typically in separate compartments.

By administering non-depleting CD4 and CD8 mAbs

together, tolerance can be conferred to a primary antigen in a patient. The CD4 and CD8 mAbs can be used to treat autoimmune diseases and to prevent graft rejection without the need for long term immunosuppressive

5 chemotherapy. Tolerance to a graft such as an organ graft or bone marrow transplantation can be achieved.

There are advantages of using non-depleting mAbs in vivo. For example, injection of a short course of non-depleting mAbs allows quicker recovery of competent
10 cells from blockade and therefore may lessen the risk of opportunistic infection and other complications due to immune deficiency (e.g. leukemia relapse after T-depleted bone-marrow transplantation) following mAb treatment. In addition, it is now known that CD4 cells can be further
15 divided into different functional subsets, some of which may be involved in immune regulation. Bulk elimination of T cells may of course result in immune suppression, but it may also destroy an possible influence of CD4+ regulatory cells. Therefore, a more subtle manipulation
20 may be more beneficial for guiding the immune system to a desired state.

Tolerance may preferably be attained by administering first to a patient depleting CD4 and/or CD8 mAbs. The invention therefore further provides:

- 25 - non-depleting CD4 and CD8 mAbs and depleting CD4 and/or CD8 mAbs for use together in a method of treatment of the human or animal body by surgery or therapy, more specifically in inducing tolerance to an antigen;
- 30 - products containing a non-depleting CD4 mAb, a non-depleting CD8 mAb and either or both of a depleting CD4 mAb and a depleting CD8 mAb as a combined preparation for simultaneous, separate or sequential use in inducing tolerance to an antigen; and
- 35 - a pack comprising a non-depleting CD4 mAb, a non-depleting CD8 mAb and either or both of a depleting CD4

mAb and a depleting CD8 mAb, typically each in a separate compartment.

A combination of non-depleting CD4 and CD8 mAbs can be used to induce tolerance to any antigen without
5 the need for other immunosuppressive agents. A non-depleting mAb is a mAb which depletes fewer than 50%, for example from 10 to 25% and preferably less than 10% of target cells in vivo. They may be used to induce tolerance to a Class I antigen or to a Class II antigen
10 or to an antigen presented by a Class I or Class II antigen. They may be used to induce tolerance to both antigens. In the case of a transplant, for example, Class I and Class II major histocompatibility (MHC) antigens and non-MHC or minor histocompatibility (minors)
15 antigens may be presented. Apart from transplantation antigens, the present invention can be used to induce tolerance to globular proteins, glycoproteins such as immunoglobulins, materials carried on particles such as pollen proteins, polypeptides intended for therapeutic
20 use such as interferon, interleukin-2 or tumour necrosis factor, or hormone replacements such as leutinizing hormone, its analogues and antagonists. Further specific antigens to which tolerance can be conferred include synthetic peptide analogues of protein therapeutic agents
25 which are used for receptor block aid and alloantigens. It is alloantigens which are considered to be responsible for rejection of foreign tissue in tissue transplants or skin grafts.

The mAbs which may be used are mAbs which are
30 specific for the CD4 cell surface antigen (CD4 mAb) or for the CD8 cell surface antigen (CD8 mAb). By CD4 and Cd8 mAbs we mean not only mAbs specific for the human CD4 and CD8 surface antigens but also mAbs specific for the corresponding surface antigens in other species such as
35 the L3T4 antigen in mice which is the murine equivalent

of the human CD4 antigen. The mAbs are typically of the IgG₂ Class such as rat IgG_{2a}, mouse IgG_{2b} or human IgG₂, but may be human IgG₄. mAb fragments comprising the antibody binding site may be used, such as Fab and F(ab)₂ fragments.

The CD4 and CD8 mAbs are administered together to a host. They may be administered as part of the same formulation or as separate formulations. Typically both mAbs are administered from 1 to 7 times a week, preferably from 1 to 4 times a week, for example three times a week, for from 2 to 4 weeks, preferably for 3 weeks. An effective amount of the non-depleting mAbs is given. Testing for saturating amounts of antibody in serum should indicate that sufficient antibody is present. Enough of each non-depleting mAb is consequently administered to induce a tolerance-permissive environment in a subject under treatment. The CD4 and CD8 cells can thus be blocked.

The amount of non-depleting CD4 mAb and of non-depleting CD8 mAb administered to a patient depends upon a variety of factors including the age and weight of a patient, the condition which is being treated and the antigen(s) to which it is desired to induce tolerance. In a model mouse system from 1 μ g to 2mg, preferably from 400 μ g to 1mg, of a mAb is administered at any one time. In humans from 1 to 400mg, such as from 3 to 30mg, for example from 5 to 20mg, of antibody may be given. A CD11a mAb, a non-depleting mAb, may be used in addition to CD4 and CD8 mAbs or in place of either or both of the CD4 or CD8 mAbs.

A foreign antigen(s) to which it is desired to induce tolerance can be administered to a host from up to 5 days before a course of non-depleting CD4 and CD8 mAbs is commenced to up to 5 days or even 2 to 3 weeks after the course has been completed. Generally, however, an

antigen is administered within the first 14 days of the course commencing, typically within 7 days of the course commencing. The antigen can be given at the time the course of CD4 and CD8 mAb treatment is commenced.

- 5 Tolerance can therefore be induced to an antigen in a host by administering non-depleting CD4 and CD8 mAbs and, under cover of the mAbs, the antigen. A patient may be operated on surgically under cover of the non-depleting CD4 and CD8 mAbs to be given a tissue
- 10 transplant such as an organ graft or a bone marrow transplant. Also, tolerance may be induced to an antigen already possessed by a subject. Long term specific tolerance can be induced to a self antigen or antigens in order to treat autoimmune disease such as multiple
- 15 sclerosis or rheumatoid arthritis. The condition of a patient suffering from autoimmune disease can therefore be alleviated.

- Persistent antigen is required to maintain tolerance. A tissue graft, for example, supplies the
- 20 antigen to maintain tolerance to itself. The same applies to self (auto) antigens in the treatment of autoimmune diseases. In the case of extraneous foreign antigen such as allergens, antigen reminders can be given at regular intervals.

- 25 It may be preferable to treat a host with a depleting CD4 mAb and/or a depleting CD8 mAb before commencing treatment with non-depleting mAbs. A depleting mAb is a mAb which depletes more than 50%, for example from 90 to 99%, of target cells in vivo.

- 30 Depleting antibodies include rat IgG_{2b} or IgG₁, mouse IgG_{2a} and human IgG₁ and IgG₃. A depleting CD4 mAb and/or a depleting CD8 mAb may therefore be used to reduce the relevant population of T cells. The non-depleting mAbs therefore have fewer T cells to work on.

- 35 Depletion may alternatively be achieved by conventional

immunosuppressive therapy such as administration of another mAb such as CAMPATH (Trade Mark) 1, steroids, cyclosporin, ALG (anti-lymphocyte globulin) or irradiation.

5 The level to which a population of T cells is reduced by a depleting mAb may depend upon the antigen(s) to which it is wished to achieve tolerance. It may be preferable to reduce the T cell population further for difficult antigens, for poorly matched tissue grafts,
10 where recipients have been previously exposed to donor antigens and are primed (for example multiply transfused patients) or in autoimmune diseases where the patients are both primed and undergoing continuous activation of their autoimmune state. A poor tissue match, for
15 example, is where one or more Class I major histocompatibility antigens do not match.

 It may therefore be necessary to reduce a population of CD4-positive helper T cells to less than about 70%, for example less than about 50%, 20% or even
20 10%, of their normal level. The more difficult it is likely to be to achieve tolerance, the greater the amount of depletion it is desirable to achieve. CD8-positive T cells may be depleted in the same way.

 The depleting CD4 mAb and/or depleting CD8 mAb is
25 typically administered from 1 to 7 times a week, preferably from 2 to 4 times a week, for example three times a week or once or twice, preferably once, from 1 to 7 days, for example from 1 to 5 days, before commencement of the treatment with non-depleting CD4 and CD8 mAbs. An
30 antigen to which it is desired to induce tolerance may be administered at the same time as, or within 5 days of, administration of the depleting mAb. The amount of depleting mAb which is given depends upon a variety of factors including the level to which it is desired to
35 reduce the relevant population of T cells, the age and

weight of a patient, the condition which is being treated and the antigen(s) to which it is desired to induce tolerance. In a mouse model system, dose of from 1 μ g to 2mg, preferably from 400 μ g to 1mg, of a depleting mAb may be given. In humans, from 1 to 400mg, such as from 3 to 30mg, for example from 5 to 20mg, of antibody may be given.

The depleting and non-depleting CD4 and CD8 mAbs can be raised in any convenient manner. They may be made by conventional methods of fusing immune rat spleen cells to a rat myeloma cell line such as Y3/Ag 1.2.3. (Clark and Waldmann, chapter 1 of "Monoclonal Antibodies", which is a book edited by P.C.L. Beverley in a series "Methods in Hematology", Longman (Churchill Livingstone), 1986). Any other method may be employed such as by fusing immunised mouse spleen cells with a mouse myeloma, a rat myeloma or human myeloma or by recombinant DNA methodologies.

All mAb administrations are given parenterally, for example intravenously. The mAbs are generally being given by injection or by infusion. For this purpose a mAb is formulated in a pharmaceutical composition containing a pharmaceutically acceptable carrier or diluent. Any appropriate carrier or diluent may be used, for example isotonic saline solution. When specific antigens to which it is wished to confer tolerance are given, these may be administered parenterally, for example intravenously, intramuscularly or subcutaneously. The antigen is typically formulated with a pharmaceutically acceptable carrier or diluent as above.

The respective hybridomas producing mAbs YTS 177.9, YTS 191.1, YTS 105.18 and YTS 169.4 were deposited at the European Collection of Animal Cell Cultures, Porton Down, G.B. on 30 May 1990 under accession numbers ECACC 90053005, ECACC 87072282, ECACC 90053004 and ECACC

87072284.

The following Examples illustrate the invention.
In the accompanying drawings:

- Figure 1 shows synergy and interference of complement lysis by combinations of rIgG2a and rIgG2b Mabs. CBA/Ca thymocytes labelled with $^{51}\text{-Cr}$ ($30\mu\text{Ci/ml}$) were incubated with Mabs as indicated under each bar and 2% guinea pig serum as the complement source. Mab concentration: (A) $5\mu\text{g/ml}$; (B) $25\mu\text{g/ml}$ of the Mabs used.
- Figure 2 shows the results of injection of rIgG2a CD4 in vivo without T cell depletion. ATX CBA/Ca mice ($n=3$) were given a single injection of the indicated Mabs (2mg/mouse) and bled at 1, 4 and 8 weeks later. BPL were stained with biotinylated Mabs against CD4 (YTA 3.1 \odot), CD8 (YTS 156.7 \blacktriangle) and Thy-1 (YBM 29.2 \blacktriangledown), followed by streptavidin-FITC. The results were analysed by flow cytometry and the percentage of each population was calculated using that of the untreated control on the test day as 100%.
- Figure 3 shows how tolerance to HGG was induced by IgG2a CD4 Mab YTS 177.9. Normal CBA/Ca mice were given YTS 177.9 or YTS 191.1, at doses indicated on days -1, 0 and 1. 1 mg heat-aggregated HGG was injected on day 0. The mice were rechallenged with 0.5 mg HGG on days 28 and 35. Control mice received 1 mg of YTS 177.9 as others, but immunized with HGG only on days 28 and 35. Serum anti-HGG titres were measured on day 45 by an ELISA assay.
- Figure 4 shows the results of injection of YTS 177.9 to induce specific tolerance to rat IgG2a. Normal CBA/Ca mice were given 3 injections of YTS 177.9 or YTS 191.1 at the indicated doses on 3 consecutive days. 6 weeks later, they were rechallenged with weekly injection of YTH 35.4 (IgG2a rat anti-human) and Campath (Registered Trade Mark) 2 (IgG2b rat anti-human), first

in complete, then in incomplete Freund's adjuvant. On the tenth week, they were bled and anti-IgG2a (open-bar) and anti-IgG2b (cross-hatched bar) titres were measured with an ELISA assay using plates coated with HPLC-purified rat IgG2a and IgG2b mAbs respectively.

Figure 5 shows prolonged allogeneic skin graft survival after IgG2a CD4 and CD8 Mab treatment. Normal CBA/Ca mice (n=6) were given 3 injections of YTS 177.9 and YTS 105.18 or YTS 191.1 and YTS 169.4 (total antibody, 3 mg/mouse) on days 0, 2 and 4. Full thickness BALB/c tail skin was grafted on day 0. Statistic value by Logrank method: IgG2b Mab treated (\blacktriangle) v.s. non-treated control (\bullet) $p < 0.005$; IgG2a treated (\blacktriangledown) v.s. control $p \leq 0.001$; v.s. IgG2b treated $p \leq 0.03$.

Figure 6 shows tolerance to allogeneic skin grafts by rIgG2a mAbs. Normal CBA/Ca mice were grafted with B10.BR skin and received (1) a mixture of YTS 177.9 and YTS 105.18 on days 0 and 2 (2 mg/mouse total); (2) the same mAbs over 3 weeks (9 mg/mouse total). On day 89, they were regrafted with skin from B10.BR (3 and 4) and B10.D2 (dotted lines).

Figure 7 shows the results of experiments investigating tolerance to bone marrow and skin grafts. A: Tolerance to B10.BR. Recipient CBA/Ca mice (n=8) were given 1-3 weeks injections of a combination of rIgG2a CD4 and CD8 mAbs, following an infusion of 10^7 T-depleted spleen cells and 10^7 bone marrow cells. Control mice (n=4) were either bone marrow and saline injected or bone marrow treated only. All groups were grafted with B10.BR skin thirty days later. Chimerism of donor-allotype Ig was measured at 24 and 60 days post bone marrow transplant as 2.6 ± 1.6 and 1.3 ± 0.7 per cent respectively for marrow and antibody treated mice (\bullet). Neither saline (\blacktriangle) nor BM only (\blacksquare) groups showed any detectable chimerism. B: Tolerance to AKR/J. Normal

CBA/Ca recipient mice were injected for 3 weeks with a combination of rigG_{2a} CD4 and rigG_{2b} CD8 mAbs. 2×10^7 AKR/J bone marrow cells were infused intravenously 2 days after the beginning of mAb treatment. 4 weeks later, they were grafted with AKR/J skin (●). Control mice were antibody-treated only (▲).

Figure 8 Tolerance to skin grafts can be established in ATx mice. ATx mice (n=9) were injected with rigG_{2a} CD4 and CD8 mAbs every other day for 2 weeks. B10.BR skin was grafted 3 days after the beginning of mAb treatment. On day 60, they were regrafted with B10.BR and BALB/c grafts (●) were rejected by ATX mice carrying B10.BR skin grafts (▲). Control ATx mice were grafted with B10.BR skin (■).

Figure 9 shows allogeneic skin graft survival in mice treated with difference combinations of CD4 and CD8 antibodies. Recipient CBA/Ca mice, in three groups of 8-14, were grafted with BALB/c skin on day 0, and treated with monoclonal antibodies from day 0 to day 2, as described in Example 2. The first group (▼) received only rat IgG_{2a} antibodies, the second (◆) received a cocktail of rat IgG_{2b} antibodies, while the third group (●) was given the combination protocol of rat IgG_{2b} followed by rat IgG_{2a} antibodies. All mice in this third group were given new BALB/c (▲) and B10 (■) skin grafts at day 94 without further antibody.

Analysis of graft survival: IgG_{2a} group MST=28 days versus IgG_{2b} group MST=55 days; $P < 0.006$: Combined protocol group original BALB/c graft MST=121 days compared to IgG_{2a} or IgG_{2b} groups $P < 0.001$: Combined protocol group second BALB/c graft MST=43 days compared to third party B10 graft MST=16 days $P < 0.04$.

Figure 10 shows the induction of tolerance to MHC-mismatched skin grafts. Groups of 8-12 CBA/Ca mice were grafted with B10 skin on day 0, and treated with

monoclonal antibodies from day 0 to 21, as described in Example 2.

- (a) The first group were control mice (▼) given no antibody. A second group (♦) were given only rat IgG_{2a} antibodies, while the remaining group (●) all received the combined IgG_{2b} and IgG_{2a} protocol. This last group were given new B10 skin grafts (▲) and third party BALB/c skin (■) on day 119.

Analysis of graft survival: control (no antibody) group MST=14 days compared to IgG_{2a} group MST=48 days $P<0.001$: Combined protocol original B10 grafts MST>250 days compared to second B10 grafts MST=44 days (from day 119) $P<0.002$: second B10 grafts compared to third party BALB/c grafts MST=13, $P<0.003$.

- (b) A group of 8 CBA/Ca mice were given B10 skin grafts together with the combined IgG_{2b} followed by IgG_{2a} antibody protocol (●). The mice were given second B10 skin grafts on day 91 (▲) together with third party B10.BR skin (■).

Analysis of graft survival: all groups MST>200 days.

- Figure 11 shows the induction of tolerance to multiple minor antigen mismatched skin. Two groups 8 and 10 CBA/Ca mice were given AKR/J skin grafts on day 0. Control mice (▼) received no antibody. The remaining group were given the combined rat IgG_{2b} followed by rat IgG_{2a} antibody protocol (●), and were regrafted at day 122 with fresh AKR/J skin (▲) and third party minors different B10.BR skin (■).

Analysis of graft survival: no antibody controls MST=13 compared to combined protocol original AKR/J grafts MST>250 days, $P<0.001$: Second AKR/J grafts MST>128 days compared to third party B10.BR grafts MST=27 days, $P<0.009$.

- Figure 12 shows the induction of tolerance to

multiple minor antigen mismatched skin in primed recipients. Recipient CBA/Ca mice were primed to donor minor antigens by ip injection with 10^7 irradiated (2000 rad) spleen cells from either AKR/J (a,b) or B10.BR (c,d), three weeks prior to skin grafting. Groups of 5-13 mice were grafted with AKR/J (a,b) or B10.BR (c,d) skin (●) under cover of monoclonal antibodies from day 0-21, as described in Example 2. Two groups received the combined IgG_{2b} and IgG_{2a} protocol (a,c), and two were given rat IgG_{2a} antibodies only (b,d). Mice were regrafted on day 89 (a,c) or day 96 (b,d) with second donor-type (▲) grafts (AKR/J in groups a and b; B10.BR in groups c and d) and third party minors different (■) skin (B10.BR in groups a and b; AKR/J in groups c and d).

Analysis of graft survival: (a) combined protocol original AKR/J grafts MST>225 days compared to third party B10.BR grafts MST=41 days (from day 89), $P<0.007$. (b) IgG_{2a} treated original AKR/J grafts MST>200 days compared to third party B10.BR grafts MST=42 days (from day 96), $P<0.02$. (c) all groups MST>day 225 of experiment (d) all groups MST>day 200 of experiment.

Figure 13 shows the induction of tolerance in mice actively rejecting a first skin graft. Two groups of 6 and 11 CBA/Ca mice were grafted with AKR/J skin on day 0, without any monoclonal antibody treatment. On day 14, when roughly half the mice had rejected these first grafts (●), second AKR/J skin grafts were given in a fresh graft bed and monoclonal antibody treatment initiated (▲). Mice then received either the combined rat IgG_{2b} followed by IgG_{2a} three week protocol (a) or rat IgG_{2a} antibodies alone (b).

Analysis of graft survival: (a) first AKR/J grafts MST=12 days compared to second, combined protocol treated AKR/J grafts MST>92 days, $P<0.004$. (b) second

IgG_{2a} treated AKR/J grafts MST>75 days, compared to all first grafts in (a) and (b) MST=12 days; $P<0.003$.

EXAMPLE 1

MATERIALS AND METHODS

5

Experimental Animals

CBA/Ca, B10.BR and BALB/c mice were bred and kept in the conventional animal facility in the Department of Pathology, University of Cambridge, and were used in age- and sex-matched groups.

10 Identification of Monoclonal Antibodies by Flow Cytometry

A rat T-cell line NB2-6TG (kindly provided by Dr. J. Howard) was transfected with the mouse CD4 gene (Gorman *et al*, PNAS USA 84, 7644, 1987). This line, NB2.L3T4.Hyg/2.1 was used to screen new CD4 mAbs from a
15 rat anti-mouse thymocyte fusion. Cells ($1-5 \times 10^6$) were incubated with test supernatants, followed by FITC-conjugated rabbit anti-rat Ig serum (Dako, Glostrup, Denmark). The fluorescence staining was analysed by flow cytometry on a Cytofluorograph (model 50-H Ortho,
20 Westwood, MA, USA) and data were processed by an Ortho 2150 computer.

For competitive binding of mAbs, L3T4/Hyg 2.1 cells were incubated firstly with biotinylated mAbs ($1 \mu\text{g}/10^5$ cells) on ice in the presence of $10 \mu\text{g}$ unlabelled
25 mAbs for 30 minutes. Streptavidin-FITC (Amersham, U.K.) was added for a further incubation of 15 minutes. The results were analysed by the Ortho Cytofluorograph.

Two-colour fluorescence staining was carried out by serially incubating mouse spleen cells with
30 biotinylated first mAb, streptavidin-phycoerythrin (Serotech, Kidlington, U.K.), second mAb and FITC-conjugated, isotype specific mAbs. The green and red fluorescence was detected and displayed on a log scale by the Ortho Cytofluorograph.

Immunofluorescence staining of peripheral blood
lymphocytes (PBL)

Mouse PBL were separated by centrifugation on Ficoll (specific density 1.079, Phamacia, Sweden) at 3000 x g for 20 minutes. Cells at the interface were collected and washed in PBS/BSA/azide. They were then stained either with rIgG_{2b} mAbs in tissue culture supernatant, followed by FITC conjugated NORIG 7.16 (anti-rIgG_{2b}) or with biotinylated mAbs and followed by FITC-streptavidin (Amersham). The results were analysed by flow cytometry as above.

Complement lysis

Complement lysis was performed as described previously. In brief, mAbs were diluted and incubated with ⁵¹Cr labelled mouse thymocytes and 2% guinea pig serum added as the complement source. After 45 minutes at 37°C, the supernatant was collected and radioactivity measured by a Philips Automatic Gamma Counter (Philips, Eindhoven, The Netherlands). The specific lysis was calculated as

$$\% \text{ of lysis} = (e - s) / (t - s) \times 100,$$

where e is the sample count, s is the spontaneous release and t is the total count.

Induction of Tolerance to Human Gamma Globulin (HGG)

The method of tolerance induction to HGG has been described previously (Benjamin et al, J. Exp. Med 163, 1539, 1986). Normal CBA/Ca mice were injected with CD4 mAbs intravenously (i.v.) on day 1, intraperitoneally (i.p.) on day 0 and 1. 1mg of heat-aggregated HGG was given i.p. on day 0. The mice were re-challenged with 0.5mg HGG on days 21 and 31. On day 38, the IgG anti-HGG response was measured by ELISA.

Detection of Mouse Antibody Responses by ELISA

To measure murine antibody responses, sample sera were serially diluted in flat-bottom flexible plates

(Becton Dickinson, USA) coated with purified protein antigens and incubated for 30 minutes. The plates were washed with PBS/0.05% Tween 20 (Sigma, Poole, UK) and then incubated with biotinylated sheep anti-mouse IgG (Amersham) for 30 minutes, and washed as above. Streptavidin-horse radish peroxidase (Amersham) was added in the plates for 15 minutes and after another 3 washes, the reaction was developed by 5mg/ml o-phenylenediamine and 0.1% hydrogen peroxide. The absorbence at 490nm was read and titres determined by comparison with a standard positive control.

Skin Transplantation

Skin grafts were transplanted by a modified method of Billingham *et al* (Nature 172, 603, 1953). Recipient mice were anaesthetised with 1µg/mouse Hypnodil and 0.2µg/mouse Sublimase (Janssen, Oxford, UK) via i.p. injection. Full-thickness skin graft from donor tail (0.5cm x 0.5cm) was transplanted onto the graft bed on the lateral thoracic wall, covered with vaseline gauze, cotton bandage and protected by plaster for 7 days. The graft survival was observed and recorded daily thereafter, the end point of survival being the last day when no live graft tissue could be seen.

The median survival time (MST) was calculated and analysed by the Logrank method (Peto *et al*, Br. J. Cancer 35, 1, 1977). Mice were thymectomised at 4 weeks of age by the method of Monaco *et al* (J. Immunol, 96, 229, 1966) and used at least 4 weeks later. All were checked at sacrifice and none were found incompletely thymectomised.

Preparation of bone marrow cells for transplantation

Bone marrow cells were harvested from donor mice pretreated with CD4 and CD8 depleting antibodies to remove T-cells. 2×10^7 live cells were transferred into suitably prepared recipients to determine whether tolerance could be induced. Chimerism could be

determined by measurement of donor Ig allotype or donor Thy-1.1 allotype as described previously (Qin *et al*, 1989, J.Exp. Med. 169, 779).

Adoptive transfer of spleen cells

5 Donor spleen cells were obtained from either naive CBA/Ca mice or CBA/Ca mice that had been presensitized to B10.BR spleen cells and skin grafts 4-8 weeks previously. These were transferred into tolerant CBA/Ca recipients that had been treated with mAbs,
10 grafted with B10.BR skin and had carried the grafts for more than 200 days. Each recipient mouse was injected intravenously with 5×10^7 spleen cells. As controls, adult thymectomised (ATx) mice were treated with rIgG_{2b} CD4 and CD8 mAbs to deplete CD4+ and CD8+ cells. Such
15 mice are unable to reject allogeneic grafts (Cobbold *et al*, 1984, Nature, 312, 548) and were used here to demonstrate that the transferred cells could function.

Mixed lymphocyte culture

Responder spleen cells were washed and
20 resuspended in Iscove's modified Dulbecco medium (IMDM) supplemented with 10% heat-inactivated human AB serum. Stimulator spleen cells were treated with 25µg/ml mitomycin-C (Sigma, Poole, U.K.) at 37°C for 30 minutes and washed twice in IMDM. 3×10^5 responder cells and
25 3×10^5 stimulators were cultured in 96-well flat-bottom microtitre plates at 37°C with 5% CO₂. On day-4, cells were pulsed with 10µCi/ml ¹²⁵I-iodo-deoxyuridine (Amersham) for six hours. Incorporation was measured in a gamma counter. Geometric means of triplicates were calculated.

Stimulation of lymphocytes with monoclonal antibodies

30 The mitogenic anti-Vβ6 mAb, 46-6B5 was kindly given by Dr. H. Hengartner (Payne *et al*, 1988, PNAS USA 85, 7695). The mAb was coupled onto 96-well flat-bottom plates at
35 1µg/ml at 37°C overnight, then rinsed extensively with

IMDM before use. The CD3 mAb, 145-2C11, was a kind gift from Dr. J. Bluestone (Leo *et al*, 1987, PNAS USA 84, 1374), and was used at 1:100 dilution of serum-free tissue culture supernatant. 4.5×10^5 spleen cells were added into each well, in IMDM and 10% heat-inactivated human AB serum. Proliferation after 4 days of culture was measured as above.

RESULTS

Identification of rIgG_{2a} mAb Binding to CD4 Molecule

10 A mAb, YTS 177.9, was isolated on the basis of its binding to an L3T4 transfected cell line using immuno-fluorescence staining (Table 1). Its isotype was determined by reaction with an anti-rIgG_{2a} mAb, RG 7/1.7 (Springer *et al*, Hybridoma 1, 257, 1982) by ELISA (data
15 not shown). Further analyses were carried out to compare this mAb with two other CD4 mAbs described previously. Binding inhibition studies showed that YTS 177.9 bound to the same epitope as YTS 191.1 (Cobbold *et al*, 1984, Nature 312, 548) and GK 1.5 (Dialyras *et al*, Imm. Rev.
20 74, 29-56, 1983) (both CD4 mAbs) but different to that bound by another CD4 mAb YTA 3.1 (Qin *et al*, Eur. J. Immunol, 17, 1159, 1987) which recognises a separate epitope. In two-colour flow cytometry, YTS 177.9 and YTA 3.1 stained the same population of mouse spleen cells,
25 which is distinct from those stained by a CD8 mAb, YTS 169.4 (Cobbold *et al*, 1984, Nature 312, 548).

The specificity of YTS 177.9 was also tested by specific ⁵¹-Cr release assay. Although the mAb was inefficient at lysis when used alone, it exhibited
30 synergy with the CD4 mAb YTA 3.1. (Fig. 1a). On the other hand, YTS 177.9 interfered with the lysis mediated by rIgG_{2b} mAb YTS 191.1 which in fluorescence staining was shown to be cross-inhibitory (Fig. 1b).

The rIg_{2a} CD4 mAb Does Not Deplete T cells in vivo

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To determine the effect of the rIgG2a CD4 mAb on in vivo cell depletion, we used adult thymectomised (ATX) mice because in these animals, T-cell recovery following mAb depletion is much less efficient than in normal mice, due to the absence of thymus-dependent T-cell regeneration. By monitoring the change of peripheral T cells after mAb injection, it was possible to distinguish true depletion from temporary antigenic modulation or lymphocyte re-distribution. Each mouse received 2mg of either IgG2a or IgG2b antibodies and peripheral blood lymphocytes were analyzed by flow cytometry before and at various times after treatment. As can be seen in Fig. 2, one week after completion of the IgG2b mAb therapy, about 90% of the CD4⁺ cells were depleted from the periphery. This was accompanied by a decreased percentage of total T cells and a slight increase of CD8⁺ cells. In contrast, although the IgG2a mAb treatment reduced the percentage of CD4⁺ cells, the total percentage of Thy-1⁺ cells did not change significantly, nor did that of CD8⁺ cells. This is considered to be the result of antigenic modulation of CD4 molecules following antibody binding. Four weeks after treatment, the T-cell numbers in IgG2b treated groups still remained low, whilst the YTS 177.9 treated group showed a normal level of CD4⁺ cells.

25 Induction of Tolerance to HGG under Cover
 of rIgG2a CD4 mAb

Previous work using CD4 F(ab')₂ mAb fragments has suggested that mAb facilitated tolerance to protein antigens may not need the depletion of CD4⁺ T-cells. In the present study, we investigated if the non-depleting CD4 mAb, like F(ab')₂ fragments, could have the same effect. Mice were given YTS 177.9 or YTS 191.1 in a 3-day course and one injection of HGG (0.5mg/mouse) on the second day (Day 0). When re-challenged with HGG 4 weeks

later, YTS 191.1 treated mice and mice given high dose (1mg/mouse) of YTS 177.9 had become tolerant to HGG (Fig. 3). However, mice that had received 0.1mg or less of the mAb were still able to respond to HGG.

The Ability of YTS 177.9 to Induce Tolerance
to rIgG2a mAbs

In vivo administration of xenogeneic antibodies usually elicits antiglobulin responses in the host. One of the features of CD4 mAbs is their ability to suppress this antiglobulin response and even to induce tolerance to other antibodies of the same isotype. In the present study, it was found that the rIgG2a CD4 mAb YTS 177.9 had the same property.

Mice given 0.1-1mg/mouse YTS 177.9 made no primary antiglobulin response, (titre: <1:20). However, mice given 0.01 mg/mouse YTS 177.9 did mount a weak response (1:160). Six weeks after the first antibody injection, these animals were rechallenged with 'irrelevant' (rat anti-human) rIgG2a and rIgG2b mAbs, in Freund's adjuvant to ensure adequate stimulation. The rIgG2a was YTH 34.5 which is a mAb to human CDw52 (Waldmann et al, 1985, Adv. Exp. Med. Biol. 186, 869). The rIgG2b was YTH 12.5 which is a mAb to human CD3 (Waldmann et al, 1985). Mice were bled 4 weeks later and their serum anti-rat Ig titres were measured by ELISA. Just as rIgG2b mAbs rendered mice tolerant to rIgG2b immunoglobulins, mice given a high dose of YTS 177.9 (1mg) were shown to have become completely tolerant to rIgG2a even after repeated boosts (Fig. 4). 0.1mg YTS 177.9 was insufficient to induce tolerance although it had been immunosuppressive for primary responses. Mice given 0.01mg YTS 177.9 or non-treated control showed secondary-type antiglobulin responses. Tolerance so induced was specific, as YTS 177.9 treated mice were

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still able to respond to rIgG2b immunoglobulin, while YTS 191.1 tolerant mice could still respond to rIgG2a.

Delay of Allograft Rejection by rIgG2a mAb Treatment

We found YTS 177.9 as effective as rIgG2b CD4 mAbs in its ability to prolong allogeneic skin graft survival after a short course of treatment. In the first experiment, normal CBA/Ca mice were given daily injections of either rIgG2a (YTS 177.9) or rIgG2b (YTS 191.1) for 2 weeks, starting from 3 days before allogeneic (BALB/c) skin transplantation (approx. 7mg/mouse total mAb). In YTS 177.9 treated group, the MST was significantly prolonged to 20 days (untreated control: 11.3 days; $p \leq 0.05$, Table 2). This extended graft survival is very similar to that achieved by rIgG2b CD4 mAb which depleted cells (MST 19 days).

In graft rejection across MHC Class I and II mismatches, the depletion of both $CD4^+$ and $CD8^+$ subsets has been shown to significantly improve the graft survival beyond the effects of CD4 mAbs alone (Cobbold et al, Transplantation, 42, 239, 1986). In the present study, we used another rIgG2a CD8 mAb, which also had little effect on in vivo cell depletion together with the rIgG2a CD4 mAb. This CD8 mAb, YTS 105.18 was found to synergise with the rIgG2a CD4 mAb, YTS 177.9 in further prolonging fully allogeneic skin graft survival. For the purpose of comparison, equal amount of two rIgG2b mAbs (YTS 191.1 and YTS 169.4) or the two rIgG2a mAbs were administered. A total of 3mg/mouse of the mAbs were given on days 0, 2 and 4 relative to skin grafting. The rIgG2b treated CBA/Ca mice rejected BALB/c skin with the MST of 24 days (versus control of 10.5 days, $p \leq 0.005$, Fig. 5). Surprisingly, the graft survival in the group treated with rIgG2a CD4 and CD8 mAbs was even longer than that of mice treated with rIgG2b mAbs. 3 out of 4

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rIgG2a-treated CBA mice did not reject their BALB/c skin grafts until 45 days after transplantation (MST 46.5 days; versus IgG2b treated group, $p \leq 0.03$).

Tolerance to Skin Allografts

5 Although there have been reports documenting the permanent survival of certain allografts such as heart or pancreas after CD4 mAb treatment, it has been generally found more difficult to maintain a long-term skin graft with mAb treatment alone. We have previously shown that
10 treatment with CD4 depleting mAbs allowed the acceptance of multiple minor mismatched bone marrow, and such mice were then tolerant of donor skin. In the present study, we grafted CBA/Ca mice with skin from B10.BR mice and treated the recipients with YTS 177.9 (CD4) and YTS
15 105.18 (CD8). In this H-2 compatible, multi-minor transplantation antigen mismatched combination, all the mice given a 3-week course of mAb treatment accepted the first skin for 90 days. At this time, a second donor-type skin, together with a third party (B10.D2) graft was
20 transplanted. The data in Fig. 6 shows that the first and second grafts all continued to survive indefinitely (>90+ days) while the B10.D2 skin was rejected promptly. Of the mice that had been given only 2 injections of the mAbs, 3 of 6 maintained the B10.BR skin for 90 days but
25 these were slowly rejected after the second skin graft.

Tolerance and Anergy in Mice Grafted with Multiple Minor Antigen Mismatched Bone Marrow

Injection of CBA/Ca mice with the combination of rIgG2a CD4 and CD8 mAbs permitted bone marrow engraftment
30 and tolerance to B10.BR skin (Fig. 7). A similar protocol also permitted chimerism and tolerance by AKR/J marrow (MIs-1^a) (Fig. 7). As tolerance could be achieved without depletion of CD4 cells we had the opportunity to follow the fate of V β 6⁺ CBA/Ca T-cells in the periphery

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of the recipient. 4 weeks after monoclonal antibody and marrow infusion the spleen cells of these animals were unable to respond to MIs-1^a in vitro and were poorly reactive to solid phase stimulation with a mitogenic V 6 mAb (Table 3). FACS analysis showed normal numbers of V β 6⁺ cells in the periphery (these were shown to be of recipient Thy1.2 allotype). Clearly the V β 6⁺ T-cells were anergic to stimulation. As the CD4 mAb does not deplete T-cells these were assumed to be peripheral T-cells that had been anergized.

To confirm that the thymus was not essential for tolerance of peripheral T-cells AKR/J bone marrow was again used, this time to tolerise ATx mice to MIs-1^a. Again the spleen T-cells became anergic to MIs-1^a and V β 6 stimulation (Table 4). Neither the bone marrow nor the antibody alone could reproduce this. Clearly the combination of antibodies and marrow were required to bring about peripheral T-cell anergy.

The Demonstration of Peripheral Tolerance to Skin

Allografts in Adult Thymectomised Mice

To confirm that we were looking at bone-fide peripheral tolerance induced by the skin grafts, we repeated the experiment in ATx mice. As the rIgG2a mAbs do not kill T-cells, we expected that immune functions in these mice should return to normal soon after the cessation of antibody therapy. As was found in euthymic mice, tolerance was induced to the test skin, and third party grafts were rejected promptly (Fig. 8).

The "Peripheral" Tolerant State is Refractory to Infusion of Normal Lymphocytes

In both the above skin allograft tolerant models, we asked whether the adoptive transfer of normal lymphocytes could terminate the tolerant state. 50

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million spleen cells transfused from normal donors could not restore the ability to reject in either case. A perennial problem with this type of experiment is to show that the infused cells survive and function. In part
5 this was controlled for by transferring the same number of spleen cells into T-depleted ATx mice. In this case the transferred cells were able to mediate skin graft rejection. Only "primed cells" transferred into tolerant mice could break the tolerant state (Table 5). Similar
10 results were found in the mice tolerant to HGG, where tolerance could not be broken by adoptive transfer of normal cells unless recipient CD4+ T-cells had been depleted first. Whatever the difficulties of controlling such experiments, we must conclude that the immune system
15 of tolerant animals does not allow normal virgin T-cells to express their immune potential.

EXAMPLE 2

MATERIALS AND METHODS

Mice

20 CBA/Ca, C57BI.10/Pc, BALB/c, B10.BR and AKR/J mice were bred and maintained in the conventional animal facility of the Department of Pathology, University of Cambridge. Sex matched groups of mice were used at 6-12 weeks of age.

25 Monoclonal Antibodies

All the rat monoclonal antibodies used in these experiments are listed in Table 1. Antibodies were prepared from ascitic fluid produced in Pristane-primed (DAXLOU)F₁ rats by precipitation with 50% saturated
30 ammonium sulphate followed by dialysis into phosphate buffered saline (PBS;pH7.2). The protein concentration was adjusted to 10mg/ml as estimated by OD₂₈₀ containing 2-5mg/ml of monoclonal antibody. All antibody preparations were passed through a 0.2 micron filter

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before administration to mice.

Skin Grafting

Skin grafts were performed as previously reported (Cobbold et al, 1986, Transplantation, 41, 634). In brief, donor tail skin grafts (0.5cm to 1.0cm square) were transplanted onto the lateral thoracic wall of recipient mice under anaesthesia. The grafts were covered with gauze and plaster bandage for 7 days. Graft status was documented three times per week for the first month, and approximately weekly thereafter. Differences in survival were analyzed using the Log-Rank method (Peto et al, 1977, Br. J. Cancer, 35, 1). When performing multiple re-grafts from a range of donors, these were transplanted into a single prepared graft bed, usually on the opposite flank to the original skin graft (Cobbold et al, 1986, Nature, 323, 164). Grafts were observed three times per week initially, and weekly thereafter. Median graft survival times (MST) are given as the number of days until rejection is complete, but tolerated grafts with indefinite survival times were generally in good condition with normal hair growth.

Induction of Tolerance to Skin Grafts

Skin grafts were used as the only source of antigen/ tolerogen, and were performed as described above, on the same day as the start of monoclonal antibody treatment. Monoclonal antibodies were administered three times per week from day 0 to day 21 (inclusive), with the first two injections (day 0 and day 2) intra-venous, and the remainder intra-peritoneal. The total dose of all antibodies given per injection was 2mg of ascitic immunoglobulin fraction in 0.2ml of PBS. For rat IgG2b antibodies this was 0.5mg each of YTS 191.1.2, YTA 3.1.2, YTS 169.4.2 and YTS 156.7.7 (a synergistic depleting cocktail of CD4 and CD8 monoclonal antibodies;

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(Qin *et al*, 1987, Eur. J. Immunol. 17, 1159).

In the case of rat IgG2a antibodies, 1mg each of YTS 177.9.6 (CD4) and YTS 105.18.10 (CD8) were used. For the combined depletion and blocking protocol, the synergistic
5 rat IgG2b cocktail was given i.v. on days 0 and 2 and the rat IgG2a CD4 and CD8 three times per week thereafter i.p. until day 21.

Levels of Active Monoclonal Antibody in Serum

Serum samples taken from individual mice at
10 various times during and after monoclonal antibody administration were added, 25 μ l at 1/5 dilution in PBS containing 1% w/v bovine serum albumin (BSA) plus 5% v/v heat inactivated normal rabbit serum, to 5x10⁵ CD4 or CD8 transfected cells. The CD4 transfectant was the rat NB2-
15 6TG line expressing mouse L3/T4 (Example 1) while the CD8 (Lyt-2) gene was expressed at high levels in mouse L-cells (Zamoyska, 1985, Cell, 43, 153). Bound antibody was detected using monoclonal anti-rat IgG2a coupled to FITC (MARG2A-FITC; Serotec, Oxford, UK) at the
20 manufacturer's recommended dilution, and analysis was on an Ortho 50H Cytofluorograf with 1250 computer (Ortho, Westwood, MA, USA). The mean fluorescence was compared to a standard dilution series of purified CD4 or CD8 antibody (YTS 177.9.6 or YTS 105.18.10) in normal mouse
25 serum to derive equivalent concentration values for the serum samples. Where the fluorescence showed saturating antibody, or no detectable fluorescence above background, the result was assumed to be greater than or less than the titratable range of the standard curve.

30

Preparation of Peripheral Blood Leucocytes

Mice were bled individually from the tail vein (approx. 0.1ml) into sterile tubes containing 1U heparin. The plasma was then removed after centrifugation in a

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microfuge (6000 rpm) for 2 mins. Red blood cells were lysed twice by adding 0.9ml of water for 10 secs at room temperature followed by 0.1ml of 10X phosphate buffered saline and the white cells collected by centrifugation at 5 1,000 rpm for 7 mins (Chandler et al, 1979, J. Immunol. Methods 31, 341).

Monitoring T-cell Subsets for Depletion

Peripheral blood leucocytes were prepared from individual treated mice as described above. These were 10 stained with supernatants containing monoclonal antibodies against either CD4 (YTS 191.1.2 plus YTA 3.1.2) or CD8 (YTS 169.4.2 plus YTS 156.7.7) antigens. Other monoclonal antibodies (see Table 6) were also used as controls. Bound antibody was detected using a mixture 15 of monoclonal anti-rat IgG2b-FITC (NORIG-7.16-FITC; Clark, 1986, Methods Enzymol. 121, 548) and monoclonal anti-rat kappa light chain (MAR-18.5-FITC; Lanier et al, 1982, Hybridoma, 1, 125). The fluorescence was analyzed using an Ortho 50H Cytofluorograf with 1250 computer and 20 linear amplifiers, with gating on forward and ninety degree scatters to select for live lymphocytes.

Mixed Lymphocyte Cultures

Responder cells were obtained from individual mouse blood samples (approx 0.2ml) by water lysis as 25 described above. Each sample was assayed for proliferation by splitting the cells into three U-bottom tissue culture microtitre wells (approx 4×10^5 white cells per well) containing 4×10^5 well washed, mitomycin-C (Sigma, Poole, UK; $25 \mu\text{g/ml}$) treated stimulator spleen 30 cells. The final volume of Iscove's modified Dulbecco's medium (IMDM) containing 10% heat inactivated human AB serum was 0.1ml. After 3 days at 37°C in a 5% CO_2 gassed incubator, $5 \mu\text{l}$ of ^{125}I -deoxyuridine (IUDR; $10 \mu\text{Ci/ml}$, Amersham, UK) was added for six hours. The ^{125}I

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incorporation was measured by harvesting the cells onto glass fibre filters and counting on a Philips Automatic Gamma Counter.

A Combination of Depleting and Blocking CD4 and CD8
5 Antibodies is Permissive for Tolerance to MHC-Mismatched
Skin Grafts

Example 1 has shown that three weeks of therapy with non-depleting CD4 and CD8 antibodies permitted tolerance to multiple minor incompatible skin grafts. In
10 order to establish a treatment protocol that might tolerize across strong MHC differences we compared the effects of administering depleting (rat IgG2b), non-depleting (blocking rat IgG2a) and a combination of depleting followed by blocking CD4 and CD8 antibodies to
15 CBA/Ca (H-2^k) mice grafted with BALB/c (H-2^d) skin (Figure 9). As we have previously reported, a strictly depleting protocol delayed rejection significantly, but all mice rejected within 70 days (MST = 55 days). Non-depleting antibodies were here less effective (MST = 28
20 days), but a combination of two depleting doses followed by blockade with rat IgG2a antibodies gave the longest graft survival (MST > 100 days), although most (but not all) grafts were rejected by 200 days. In this experiment, second BALB/c test grafts were given at day
25 94, together with third party B10 (H-2^b) skin. These third party grafts were rejected promptly (MST = 16 days), demonstrating that the mice had returned to immunocompetence, whilst the second BALB/c grafts survived for a median of 43 days. This shows that there
30 must have been some degree of specific unresponsiveness to BALB/c graft antigens.

Although we did not obtain complete tolerance in this strain combination, we found the same combined protocol to be fully tolerance permissive when B10 (H-2^b)

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skin was grafted onto MHC incompatible CBA/Ca (H-2^k) mice. Figure 10 shows that 6/8 recipient mice kept their original skin grafts indefinitely (> 250 days), while all mice rejected the third party BALB/c skin grafted on day 5 119 within 15 days. Second B10 grafts had substantially extended survival times (MST = 44 days), but all were eventually rejected even when the first grafts, assumed to be genetically identical, were maintained. This result is reproducible and shows clearly that the 10 tolerated first graft enjoys privilege of tenure, despite the presence of effector cells capable of rejecting the second graft. Whatever the mechanism, the original skin graft must have induced, and maintained, a state of unresponsiveness to itself. The ability of mice to 15 distinguish between the first and second B10 grafts seemed to be dependant on rejection of the third party skin, as 5/8 mice given B10.BR instead of BALB/c grafts remained tolerant to all three (Figure 10). This demonstrates that the recipients were tolerant of B10 20 minor antigens in the context of both donor MHC, which means that the graft can itself present for tolerance, and also recipient-type MHC, which must have been through reprocessing of the original graft antigens and presentation for tolerance by recipient APCs.

25 This is the first report of antibody mediated tolerance across complete MHC barriers using highly immunogenic skin grafts as the only source of tolerogen. It should be stressed that the graft itself must have provided all the antigen presentation necessary for 30 tolerance to occur.

The Combination of Depleting and Blocking Antibodies
Also Permits Tolerance to Skin Matched for MHC but
Mismatched for Multiple Non-MHC Minor Antigens

35 In Example 1 we have demonstrated that blocking with monoclonal antibodies to CD4 and CD8 was sufficient

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to obtain tolerance to skin grafts differing in multiple minor antigens (B10.BR onto CBA/Ca). In Figure 11 it can be seen that the combined depleting followed by blocking protocol was also effective with 8/10 CBA/Ca mice keeping
5 their AKR/J grafts indefinitely. Six of these mice also held second AKR/J grafts (skin grafted at day 122 without further antibody therapy), while all mice rejected third party minor mismatched skin (B10.BR). The rejection of this third party skin was, however, somewhat slower than
10 normal CBA controls (27 days compared with the normal 14; Cobbold et al, 1986, Transplantation, 41, 634). The reverse combination (B10.BR onto CBA/Ca) was also rendered tolerant using the depletion followed by
15 blocking protocol (4/5 grafts > 220 days), but 2/5 mice accepted both the third party minors difference AKR/J skin as well as the second B10.BR graft (data not shown). This presumably reflects the large number of shared minor antigens and is reminiscent of the finding of "dominant" tolerance seen in a different experimental model
20 (Zamoyska et al, 1989, Eur. J. Immunol. 19, 111).

In summary for this section, we wish to conclude that CD4 and CD8 antibodies, when used appropriately, permit tolerance to be induced across both MHC and non-MHC antigen differences, to skin grafts alone, without
25 the need for any further haemopoietic source of antigen, nor other myeloablative therapy. Clearly, skin grafts are able to present antigen directly to peripheral T-cells either for activation, which leads to rejection, or for inactivation, which instead lead to acceptance and
30 tolerance.

The Effects of Combined Rat IgG2b and IgG2b Antibodies
on Circulating T-cells

In view of the remarkable tolerogenic effects

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seen in the above experiments, it was important to assess the effects of the monoclonal antibodies on circulating CD4⁺ and CD8⁺ T-cells. We have shown previously that rat IgG2b antibodies to either CD4 or CD8 deplete their target T-cells (Cobbold *et al*, 1984, *Nature*, 312, 548). In Example 1 rat IgG2a antibodies, in the doses given, tended only to modulate the antigen from the cell surface. In the case of the combined protocol, the initial doses of rat IgG2b CD4 and CD8 monoclonal antibodies caused a reduction in the number of T-cells as expected, and then the proportion of CD4⁺ and CD8⁺ cells actually started to recover during the continued treatment with IgG2a antibodies (Table 7), although the amount of antigen expressed was much reduced (data not shown). Residual antibody was present on the T-cells throughout the administration period, making detailed measurements of CD4⁺ and CD8⁺ cells difficult. Thereafter, the proportion of T-cells in the peripheral blood remained diminished for at least one month after stopping the antibody, an effect not observed when IgG2a antibodies were given alone in equivalent doses (data not shown).

One mechanism for the suppression of graft rejection by non-depleting monoclonal antibodies is through blocking the function of CD4 and CD8 accessory molecules on the T-cell surface during antigen presentation. This would be most effective only if serum antibody was maintained at levels sufficient to saturate antigen positive cells. The levels of active antibody in treated mice was indeed found to be sufficient to saturate the target CD4 and CD8 antigens throughout the three weeks of treatment, and in some mice up to three weeks after stopping antibody administration (Table 8). However, by day 60, there was no detectable (< 0.5ng/ml

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CD4 and < 10ng/ml CD8) monoclonal antibody left in the serum which could otherwise have maintained a non-specific immunosuppression. It should be noted that none of the mice made any detectable antiglobulin (neither
5 anti-species nor anti-idiotypic) as measured by a capture ELISA, indicating that mice were also rendered tolerant of rat immunoglobulin by this protocol.

Tolerant Mice Can Still Respond In Vitro

We found that CBA mice rendered tolerant of B10
10 skin as described above were still capable of proliferating in vitro to B10 stimulator spleen cells (Table 9), showing that a significant number of allo-reactive T-cells had not been deleted. Clearly the recipients immune system is tolerant to the antigens
15 presented by the original graft, but is still capable of reacting to in vitro presentation by spleen stimulator cells or a second skin graft.

The Induction of Tolerance in Mice Previously Primed To Minor Antigens

20 Given that virgin T-cells in the peripheral immune system could be tolerized by graft-born antigens, we sought to determine if primed T-cells were also tolerance susceptible. We found that both of the two treatment protocols (blocking alone, or depletion
25 followed by blocking) were effective for the induction of tolerance to either AKR/J or B10.BR skin in CBA/Ca mice previously primed with irradiated donor spleen cells (Figures 12a-12d). In contrast, we have previously shown that depleting the CD4⁺ and CD8⁺ T-cells, although
30 equally immunosuppressive in naive or primed mice, was not tolerance permissive for multiple minor antigen mismatched skin, as all the grafts were eventually rejected (Cobbold *et al*, 1986, Transplantation, 41, 634).

The only difference between the naive mice of

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Figure 11 and the primed mice of Figure 12 was seen after rechallenge. About half the mice in the group which had been primed and tolerized to AKR/J eventually rejected both first and second AKR/J grafts, although more slowly than the B10.BR third party grafts (MST = 63 and 21 days respectively; Figure 12a). In the B10.BR onto CBA combination the majority of mice held the original grafts indefinitely, while, in some mice, both the second B10.BR and AKR/J grafts rejected slowly (Figure 12c). This difference in behaviour of the strain combinations is compatible with that seen in naive mice (see Figure 11) and probably lies in the complex pattern of shared and unique minor antigens.

Tolerance Induction During Active Rejection
of a Skin Graft

The finding that even sensitized T-cells are tolerizable has obvious clinical implications. It would clearly be advantageous to induce tolerance in the face of an ongoing immune response, especially in autoimmunity or during an organ rejection crisis after transplantation. In Figure 13 it can be seen that it is indeed possible to reverse ongoing rejection of multiple minor antigen mismatched skin grafts and establish long-term survival, in some cases in both the first and second grafts. In this experiment, combined depletion followed by blockade was most effective (Figure 13a), but as 3/6 of the mice given the blocking (non-depleting) antibodies also held their second grafts (Figure 13b), it must be possible even for effector T-cells to be rendered inactive or tolerant.

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TABLE 1 Binding of YTS 177.9 on L3T4 transfected cells

Mab	% of positive cells L3T4 ^a
5 YTS 177.9 ^b	98.7
YTS 191.1 ^b	99.1
YTS 177.9 ^c and	1.1
YTS 191.1	
YTS 177.9 ^c and	98.1
10 YTA 3.1	

-
- a L3/T4 transfected cell lines stained with
 respective Mabs and analysed by flow cytometry.
 b Mabs were used as tissue culture supernatant
 15 followed by FITC conjugated rabbit anti-rat Ig.
 c Biotinylated Mabs, which were incubated with excess
 amount of 'cold' Mabs given underneath. The
 fluorescence was developed with FITC-streptavidin.

Table 2 Delay of skin graft rejection by IgG2a and IgG2b CD4 Mabs

MAB TREATMENT ^a	% OF CELLS ^b			GRAFT SURVIVAL (DAYS)	
	CD4 ⁺	CD8 ⁺	Thy-1 ⁺	B10.BR	BALB/c
YTS 191.1	1.9 \pm 1.5	17.3 \pm 1.7	20.2 \pm 4.4	28,29,32, 58,65.	
YTS 191.1	1.2 \pm 1.1	18.1 \pm 2.3	24.5 \pm 3.6		15,18,19, 19,20,21.
YTS 177.9	28.3 \pm 9.8	15.3 \pm 0.4	48.8 \pm 5.0	15,20,22,>120, >120,>120.	
YTS 177.9	27.5 \pm 10.1	16.1 \pm 1.2	46.9 \pm 4.2		19,19,20, 20,21,21.
none	39.8 \pm 3.0	12.5 \pm 1.5	47.0 \pm 2.8	10,11,12 12,13.	9,10,10, 11,12,12.

a) Normal CBA/Ca mice were given CD4 Mabs for 2 weeks (appr. 7 mg/mouse) starting from 3 days before skin grafting.

b) Peripheral blood collected 4 days after the last Mab injection were stained with biotinylated YTA 3.1 (CD4), YTS 156.7 (CD8) and YTS 154.7 (Thy-1), followed by streptavidin FITC. The results were analysed by flow cytometry.

c) MST of B10.BR grafts: non-treated control: 12 days; YTS 191.1 treated: 32 days (v.s. control: $p \leq 0.005$).

YTS 177 treated: 22 days (v.s. control $p \leq 0.004$, v.s. YTS 191.1 group, $p \leq 0.7$).

MST of BALB/c grafts: non-treated control: 10.5 days; YTS 191.1 treated 19 days (v.s. control, $p \leq 0.006$);

YTS 177.9 treated: 20 days (v.s. control $p \leq 0.006$, v.s. YTS 191.1 group, $p \leq 0.4$).

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TABLE 3: Tolerance of MIs-1^b mice to MIs-1^a antigen
is associated with anergy of V β 6+ cells

5	MICE	RESPONSES TO: (cpm)				
		AKR/J	BALB/c	anti-V β 6	CD3	-ve %V β 6+ cells
	1	772	11117	583	12350	596 10.2
	2	439	8964	1173	9879	460 12.1
10	3	961	6498	1050	7719	703 8.7
	4	1203	12866	1163	13496	1082 7.5
	5	859	10784	764	11230	977 7.8
	Normal	20756	9633	10440	11980	868 7.0
15	mice	27529	17253	10530	13291	642 10.2
	Marrow	38264	10042	13950	19087	815 8.1
	only	15832	8679	7267	13563	976 9.0

- 20 Normal CBA/Ca mice were treated with rIgG_{2a} CD4 (YTS 177.9) and rIgG_{2b} CD8 (YTS 156.7) for 3 weeks with a total of mAbs of 10mg/mouse. 2×10^7 AKR/J bone marrow cells were injected. 4 weeks later, spleen cells from these mice were tested in MLC and fluorescence stained for flow cytometry.
- 25 Figures given are geometric means of triplicates.

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TABLE 4: Tolerance and anergy to MIS-1^a in ATx MIS-1^b mice^a

MICE	RESPONSES TO: (cpm) ^b			% Vβ6+ cells ^c
	AKR	BALB/c	Anti-Vβ6	
1	1348	8081	696	2.8
2	1145	10231	812	2.9
3	998	7311	976	3.7
10 4	1039	6508	793	3.3
Control ^d	12463	10283	7374	3.8
Mice	15578	8864	5824	2.9

15 ^a ATx CBA/Ca mice were injected with equal amounts of
 rIgG_{2a} CD4 mAb (YTS 177.9) and rIgG_{2b} CD8 mAb (YTS 156.7)
 for 2 weeks (total mAbs 7mg/mouse). 2x10⁷ AKR bone marrow
 cells were infused 2 days after the beginning of mAb
 treatment. 8 weeks later, their spleen cells were
 20 stimulated in vitro as described in Example 2.

^b Numbers given are counts per minute (geometric means of
 triplicate samples) of each individual mouse.

^c Spleen Vβ6+ cells stained with mAb 44-22-1 (Payne et al,
 1988, PNAS USA, 85, 7695) and analysed by flow cytometry.

25 ^d Control mice were ATx CBA injected with mAbs only.

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TABLE 5: Transfer of normal lymphocytes does not break tolerance in normal mice

5	ANIMALS	CELLS INJECTED ^a	GRAFT SURVIVAL AFTER CELL TRANSFER
10	Tolerant CBA	B10.BR spleen cells	>100 days x 4
		naive CBA spleen cells	>100 days x 4
15	ATx CBA	sensitised CBA spleen cells ^c	15, 17, 17, 21,
15	T-depleted ^b naive CBA spleen cell	sensitised CBA spleen cells	12, 14, 14, 15 8, 8, 9, 10
		none	>100 days x 4

CBA/Ca mice were rendered tolerant to B10.BR skin as described in Figure 13a legend.

20 ^a Mice carrying B10.BR skin for >200 days were selected and injected with 5×10^7 spleen cells intravenously.

^b ATX CBA mice were depleted of T cells by injection of CD4 and CD8 mAbs at least 4 weeks before they were grafted with B10.BR skin.

25 ^c Sensitised spleen cells were from CBA mice which had been injected with B10.BR spleen cells and had rejected B10.BR skin grafts.

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TABLE 6: Monoclonal antibodies used

	Monoclonal antibody	Specificity	Epitope	Isotype	Reference
5	YTS 191.1.2	mouse CD4	a	IgG _{2b}	Cobbold <u>et al</u> , 1984, Nature, <u>312</u> , 548
10	YTA 3.1.2	mouse CD4	b	IgG _{2b}	Qin <u>et al</u> , Eur J. Immunol. <u>17</u> , 1159, 1987
	YTS 177.9.6	mouse CD4	a	IgG _{2a}	
15	YTS 169.4.2	mouse CD8	Lyt-2 (a)	IgG _{2b}	Cobbold <u>et al</u> , 1984, Nature, <u>312</u> , 548
	YTS 156.7.7	mouse CD8	Lyt-3 (b)	IgG _{2b}	Qin <u>et al</u> , 1989 J.Exp.Med. <u>169</u> , 779
	YTS 105.18.10	mouse CD8	Lyt-2 (c)	IgG _{2a}	
20	YTS 154.7.7	mouse Thy-1	monomorphic	IgG _{2b}	Cobbold <u>et al</u> , 1984, Nature, <u>312</u> , 548
25	YTS 121.5.2	mouse CD5	Lyt-1	IgG _{2b}	Cobbold <u>et al</u> , 1984, Nature, <u>312</u> , 548

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TABLE 7: Remaining T-cells in CD4+CD8 antibody treated mice

B10 tolerant CBA						
Per-cent of spleen cells staining positive						
Time (days)	CD4+	CD8+	Thy-1+CD5+	anti-rat Ig*	number tested	
0	34±2	16±1	47±1	0±0	4	
10 5	12±3	12±5	13±4	6±1	8	
12	18±4	18±6	18±5	3±1	3	
19	27±4	22±3	26±5	3±3	6	
45	24±5	11±2	28±4	0±0	8	

15 *Anti-rat Ig was a mixture of MAR-18.5-FITC and NORIG-7.16-FITC.

All mice received rat IgG_{2b} CD4 and CD8 followed by rat IgG_{2a} CD4 and CD8 as described in Example 2 from day 0-21.

TABLE 8: Serum levels of active IgG_{2a} CD4 and CD8 monoclonal antibodies

		CD4 serum antibody				CD8 serum antibody			
		(ng/ml)				(ng/ml)			
		[Four individual mice]				[Four individual mice]			
	Time								
	Day 0	<0.5	<0.5	<0.5	<0.5	<10	<10	<10	<10
10	Day 12	>100	>100	>100	>100	>100	120	150	>1000
	Day 19	50	>100	>100	>100	190	>1000	>1000	>1000
	Day 29	50	>100	>100	>100	75	250	>1000	>1000
	Day 32	80	90	>100	>100	500	600	>1000	>1000
	Day 36	5	100	>100	>100	60	85	500	>1000
15	Day 43	<0.5	15	70	>100	<10	<10	12	25
	Day 46	<0.5	<0.5	<0.5	1.0	<10	<10	<10	<10
	Day 60	<0.5	<0.5	<0.5	<0.5	<10	<10	<10	<10
	Day 78	<0.5	<0.5	<0.5	<0.5	<10	<10	<10	<10
20	Monoclonal antibody treatment was rat IgG _{2b} CD4 and CD8 on days 0 and 2, followed by rat IgG _{2a} CD4 and CD8 three times per week until day 21 (2mg total per injection).								

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TABLE 9: Proliferation of Peripheral Blood Lymphocytes
from B10 tolerant CBA mice

5	Proliferation (125 IUDR incorporation) to stimulators			
	Group	CBA (cpm.)	BALB/c (cpm.)	B10 (cpm.)
10	Normal CBA control	370+236	4952+5236	2210+2622
		-121	-2624	-1285
15	B10 tolerant CBA	716+800	4952+3470	3592+2875
		-448	-2091	-1656

Individual B10 tolerant CBA/Ca mice, or normal controls,
were bled from the tail vein on day 78.

Proliferation of peripheral blood cells was determined as
in Example 2.

Figures given are logarithmic means and standard deviations
of six mice per group.

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CLAIMS

1. Products containing a non-depleting CD4 monoclonal antibody and a non-depleting CD8 monoclonal antibody as a combined preparation for simultaneous,
5 separate or sequential use in inducing tolerance to an antigen.
2. Products according to Claim 1, further containing a depleting CD4 monoclonal antibody and/or a depleting CD8 monoclonal antibody.
- 10 3. A non-depleting CD4 monoclonal antibody for use in a method of treatment of the human or animal body by surgery or therapy.
4. A non-depleting CD8 monoclonal antibody for use in a method of treatment of the human or animal body
15 by surgery or therapy.
5. An antibody according to Claim 3 or 4 for use in inducing tolerance to an antigen.
6. Use of a non-depleting CD4 monoclonal antibody in the preparation of a medicament for use in
20 inducing tolerance to an antigen.
7. Use of a non-depleting CD8 monoclonal antibody in the preparation of a medicament for use in inducing tolerance to an antigen.
8. An agent for use in inducing tolerance to an
25 antigen, which agent comprises a non-depleting CD4 monoclonal antibody and a non-depleting CD8 monoclonal antibody.
9. An agent according to Claim 8, further comprising a depleting CD4 monoclonal antibody and/or a
30 depleting CD8 monoclonal antibody.
10. A pack comprising as separate components a non-depleting CD4 monoclonal antibody and a non-depleting CD8 monoclonal antibody.

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11. A pack according to Claim 10, further comprising as separate components a depleting CD4 monoclonal antibody and/or a depleting CD8 monoclonal antibody.

5 12. Non-depleting CD4 and CD8 monoclonal antibodies for use together in a method of treatment of the human or animal body by surgery or therapy.

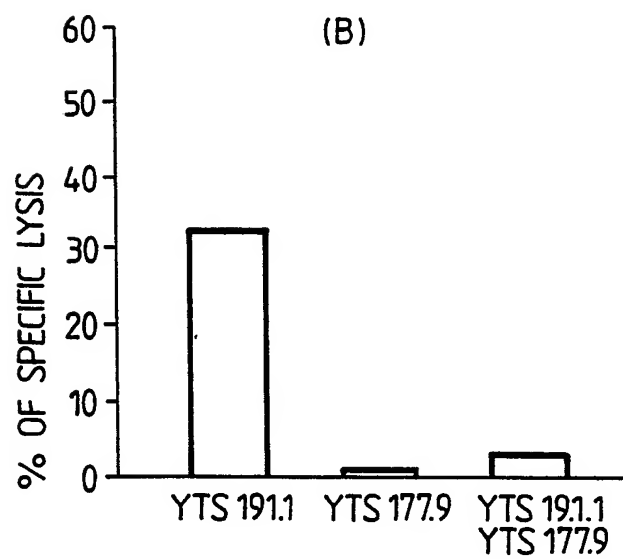
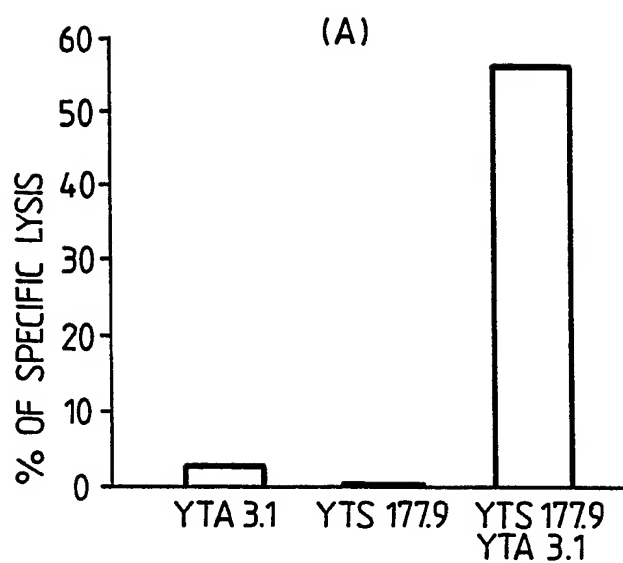
 13. Antibodies according to Claim 12, for use together in inducing tolerance to an antigen.

10 14. Antibodies according to Claim 12 or 13, further comprising a depleting CD4 monoclonal antibody and/or a depleting CD8 monoclonal antibody.

 15. A method of conferring on a subject tolerance to an antigen it is wished to administer thereto, which
15 method comprises administering to the subject an effective amount of a non-depleting CD4 monoclonal antibody and a non-depleting CD8 monoclonal antibody and of the antigen.

 16. A method of conferring on a subject tolerance
20 to an antigen already possessed by the subject, which method comprises administering to the subject an effective amount of a non-depleting CD4 monoclonal antibody and a non-depleting CD8 monoclonal antibody.

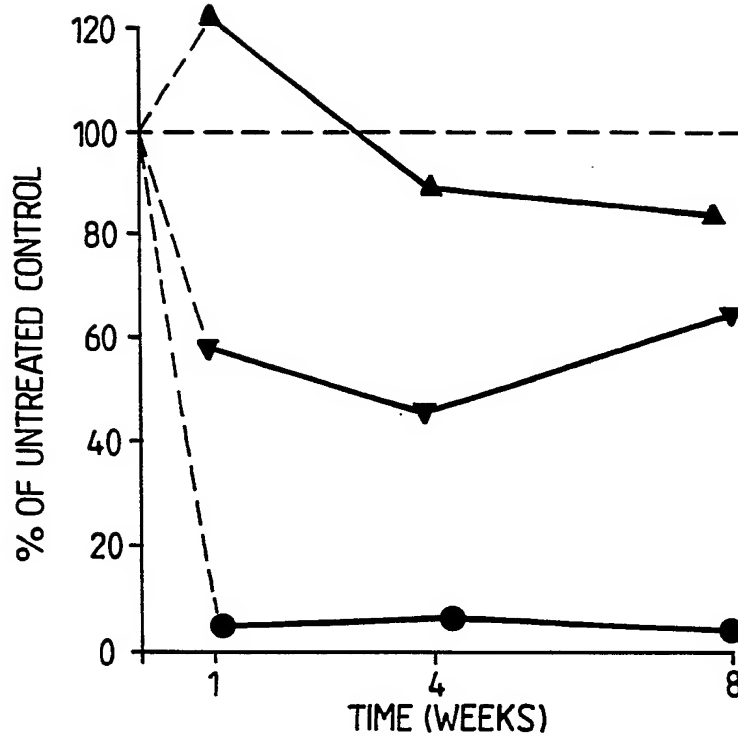
 17. A method according to Claim 15 or 16, further
25 comprising administering to the subject an effective amount of a depleting CD4 monoclonal antibody and/or a depleting CD8 monoclonal antibody prior to administration of the non-depleting CD4 and CD8 monoclonal antibodies.

Fig.1.

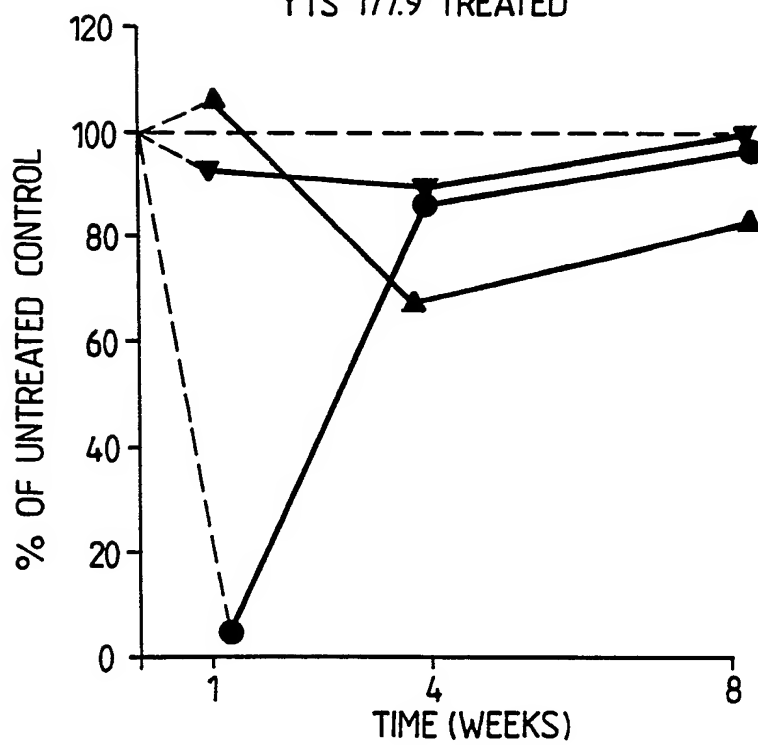
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Fig.2.

YTS 191.1 TREATED



YTS 177.9 TREATED



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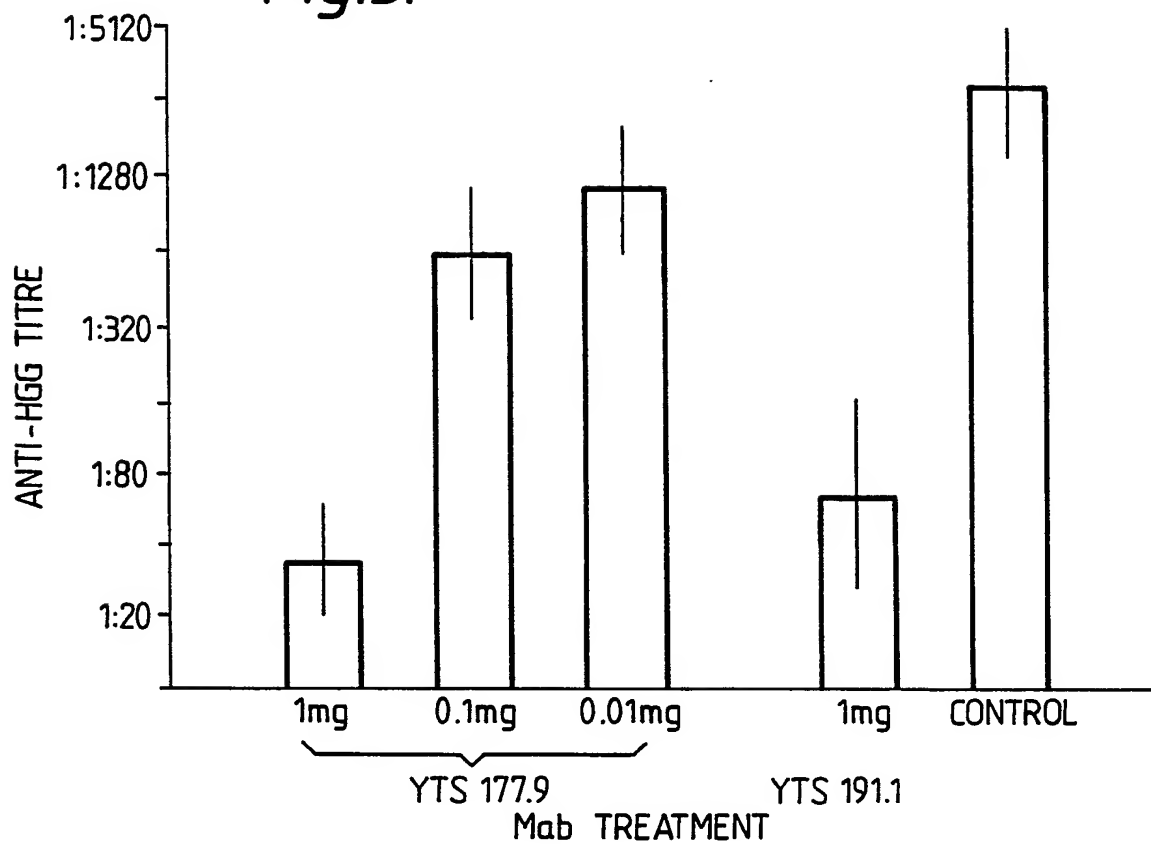
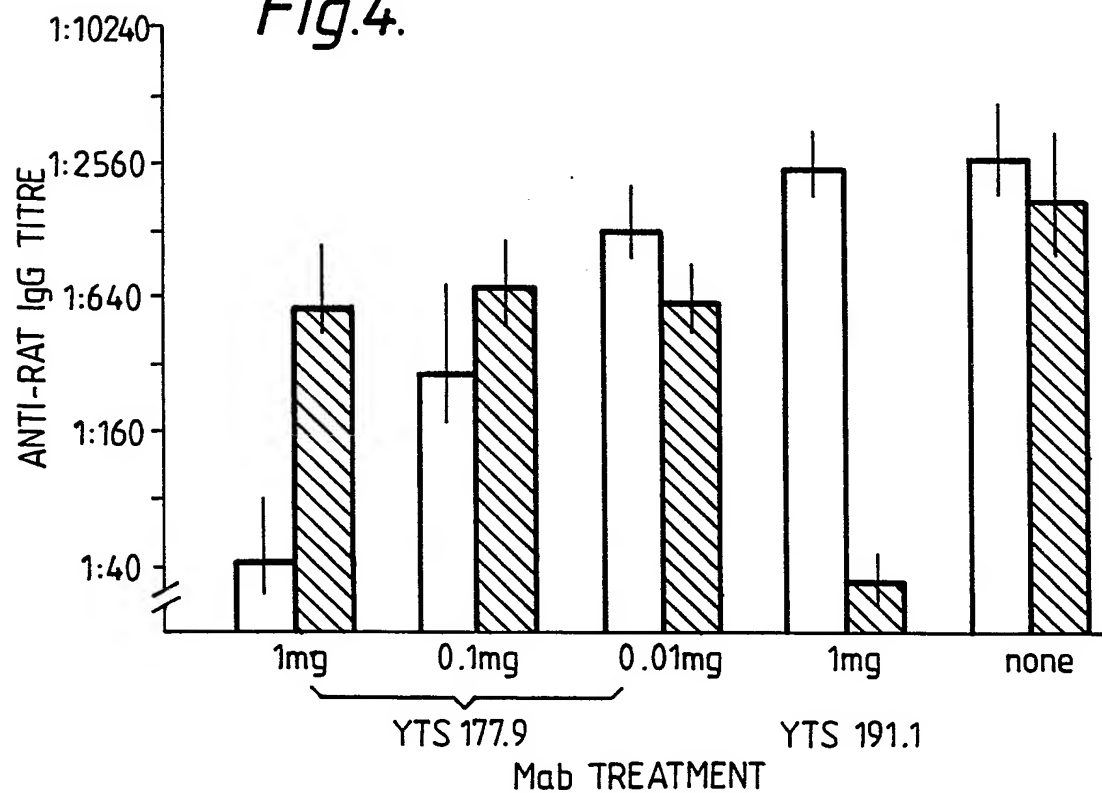
Fig.3.*Fig.4.***SUBSTITUTE SHEET**

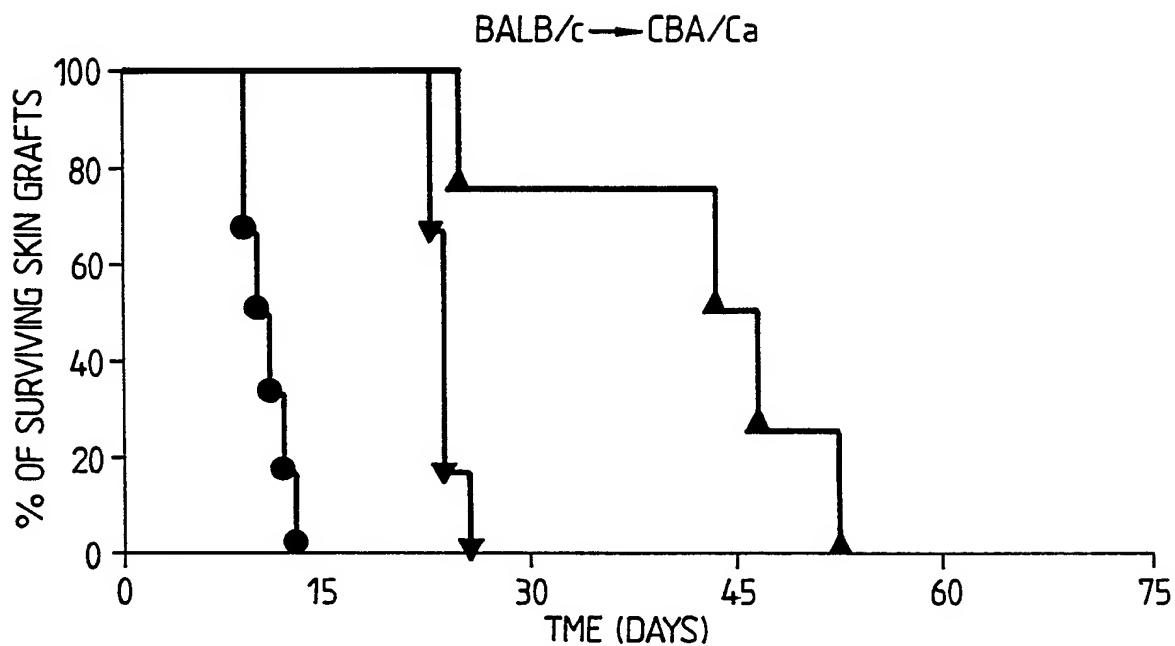
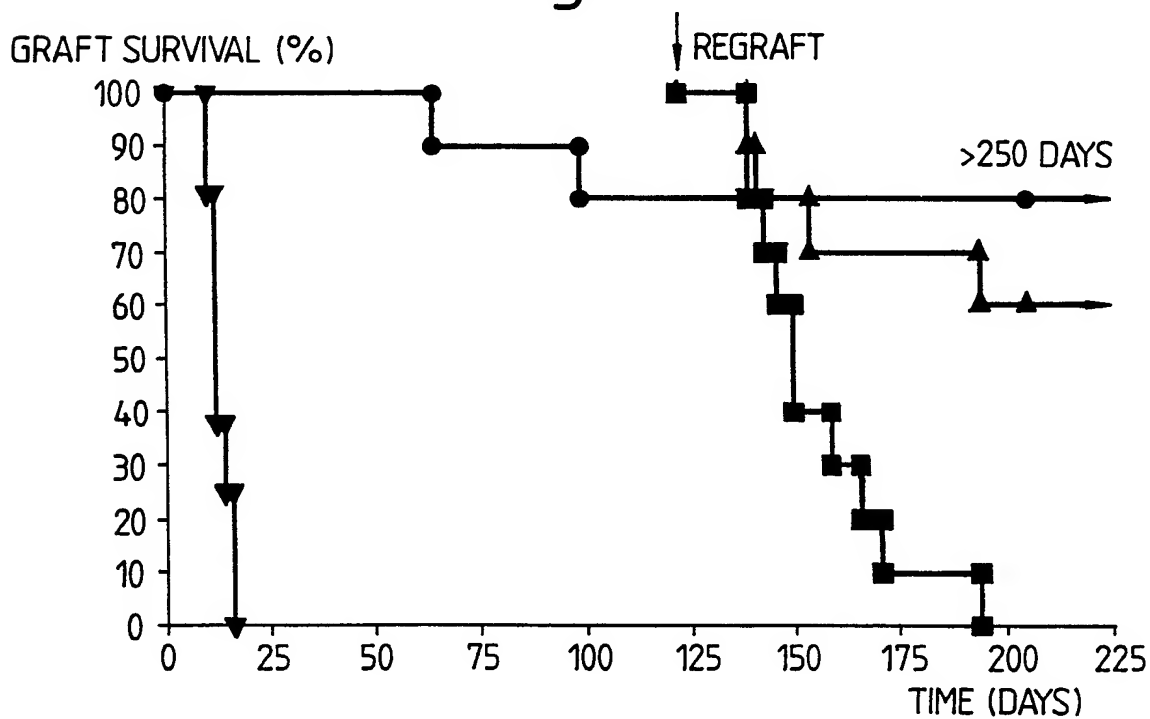
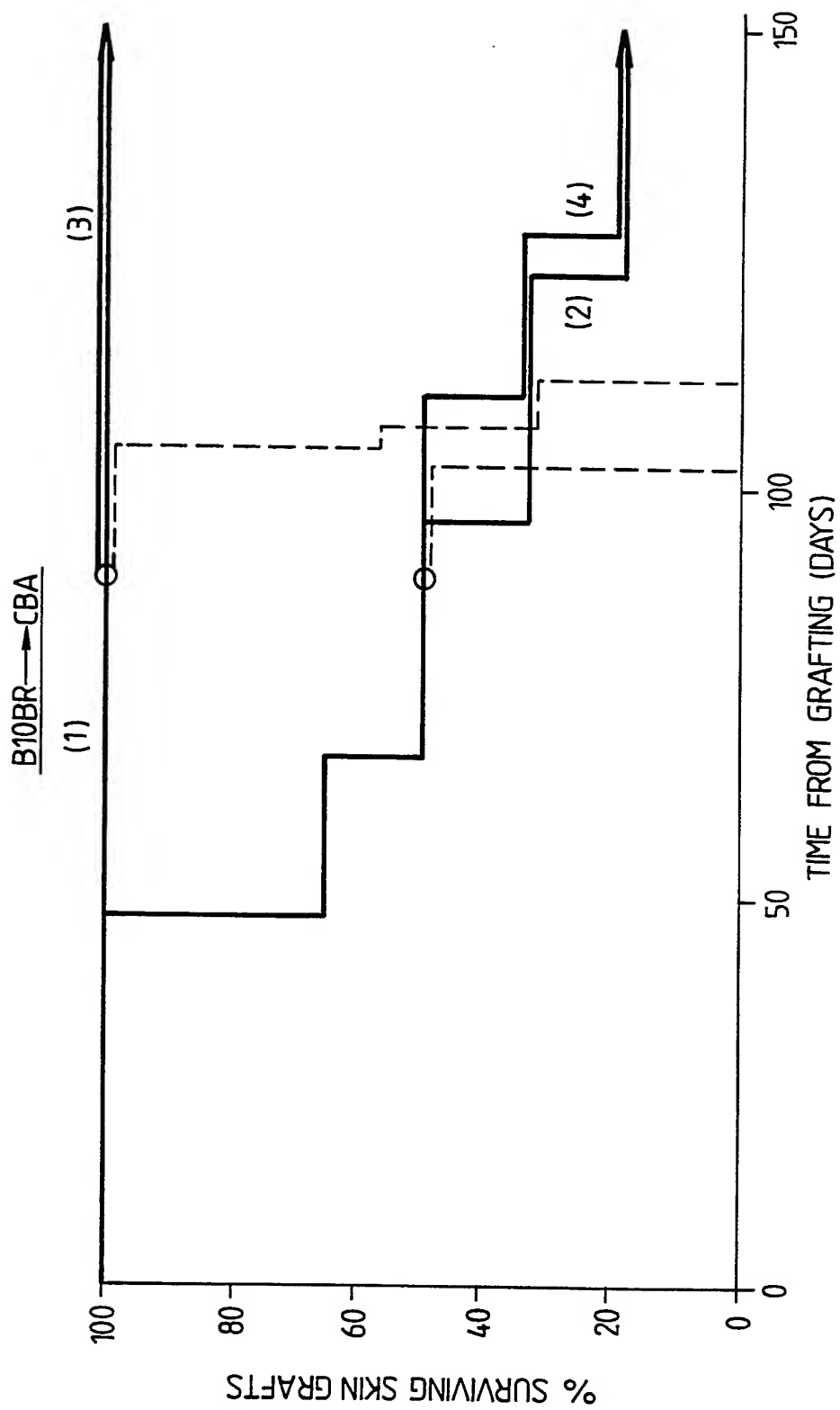
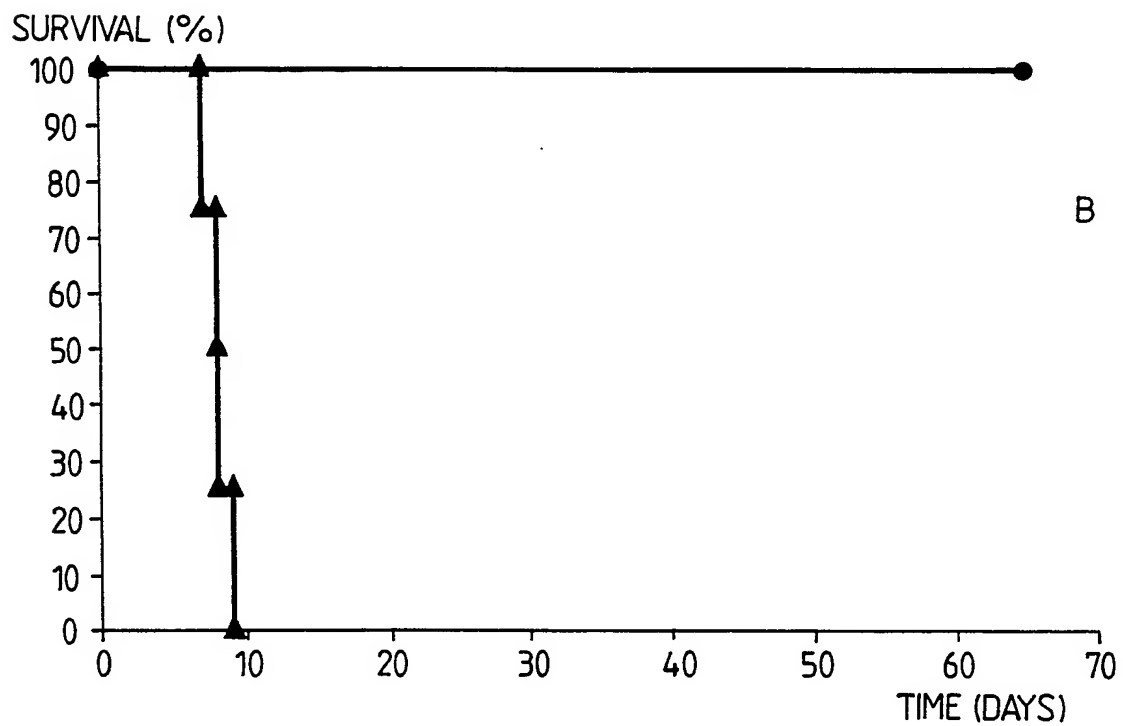
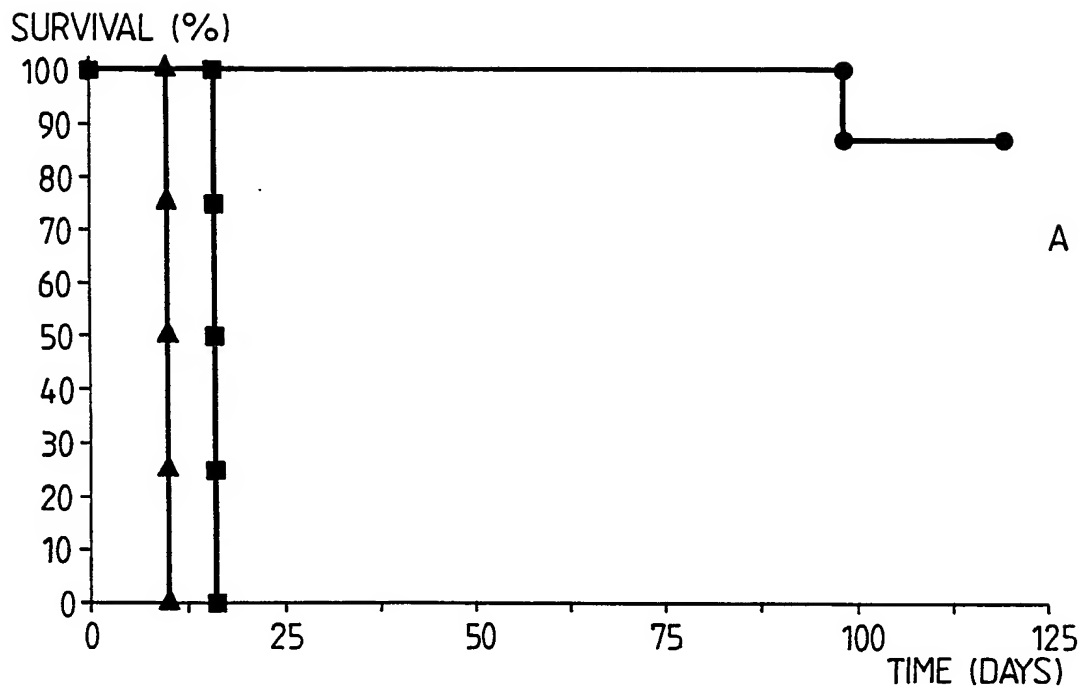
Fig.5.*Fig.11.***SUBSTITUTE SHEET**

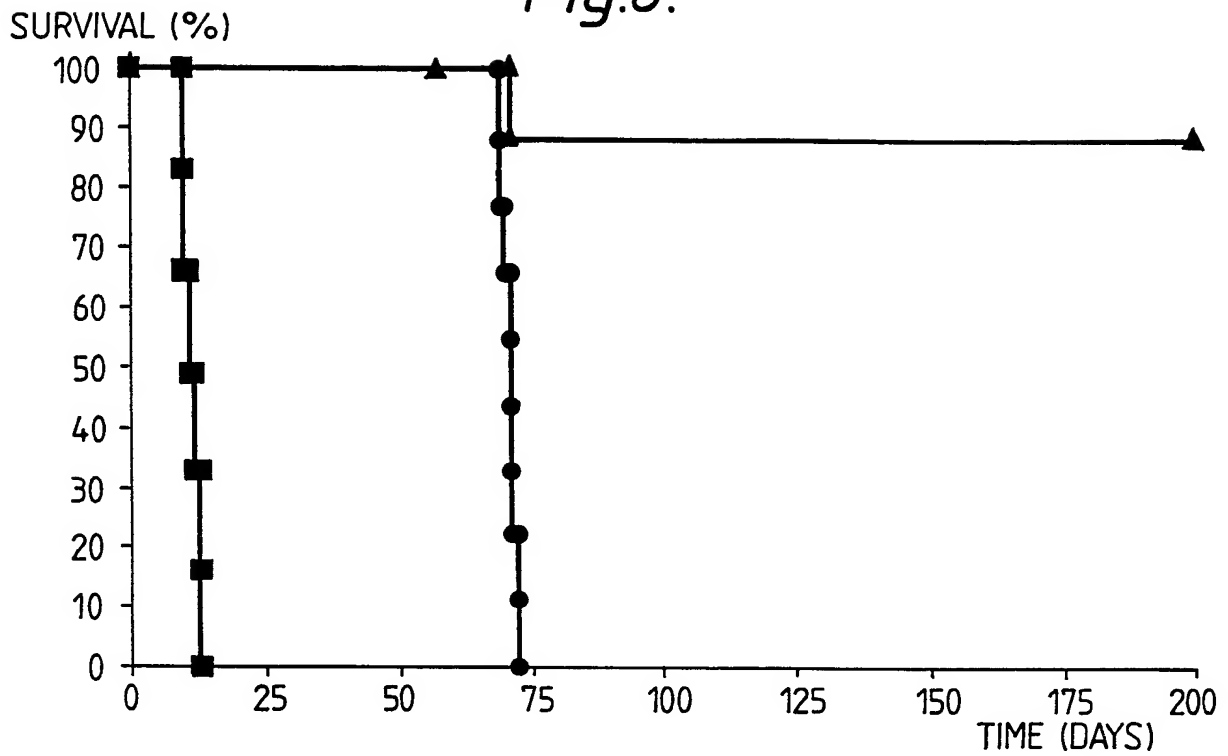
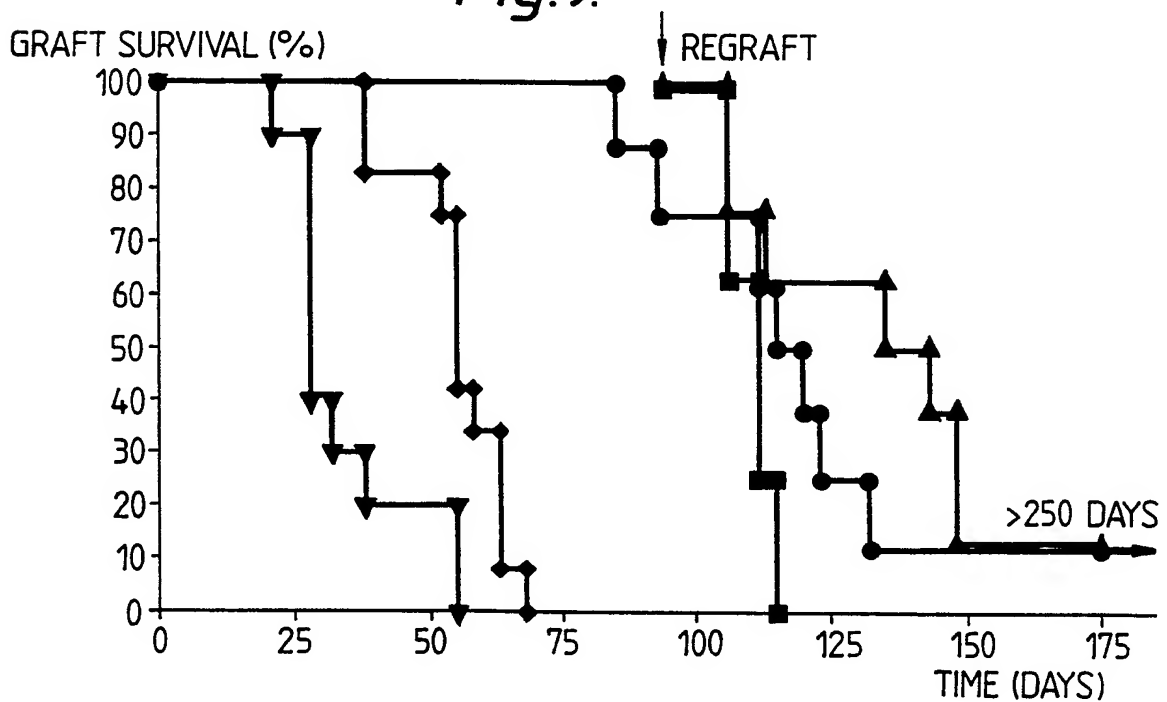
Fig.6.

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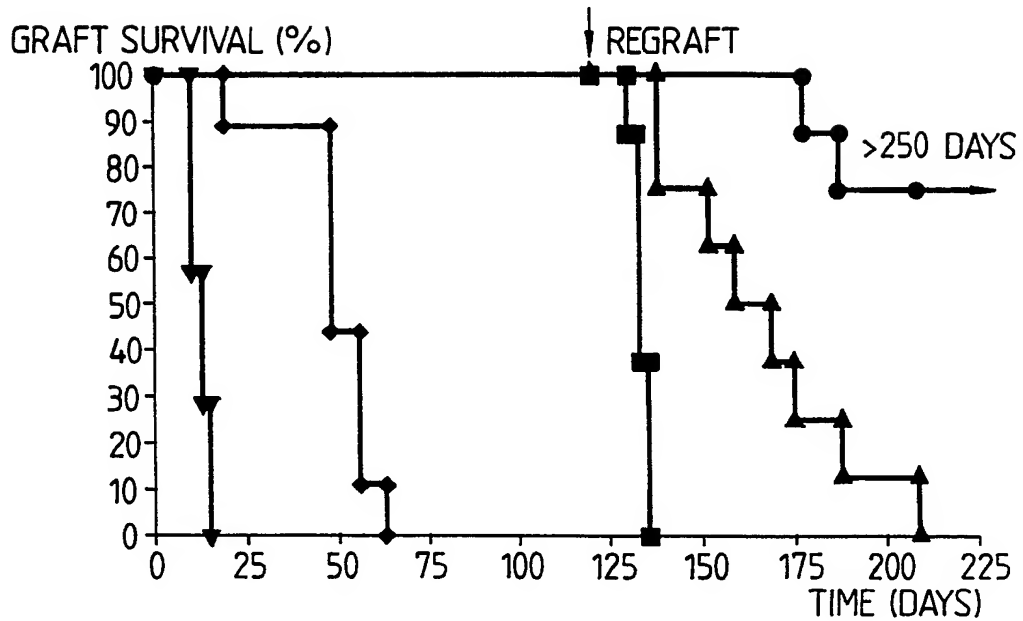
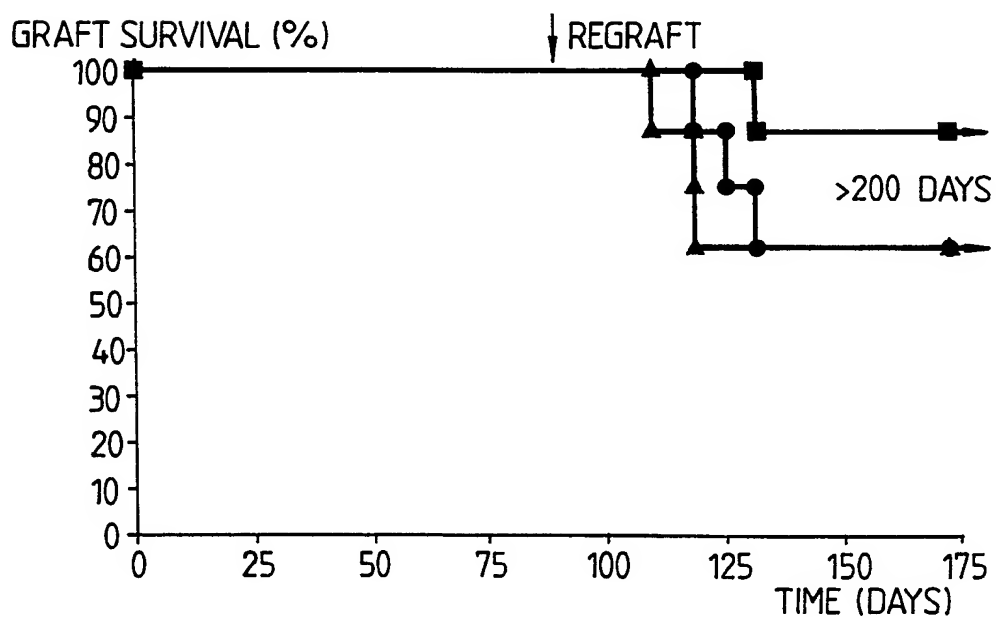
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Fig.7.**SUBSTITUTE SHEET**

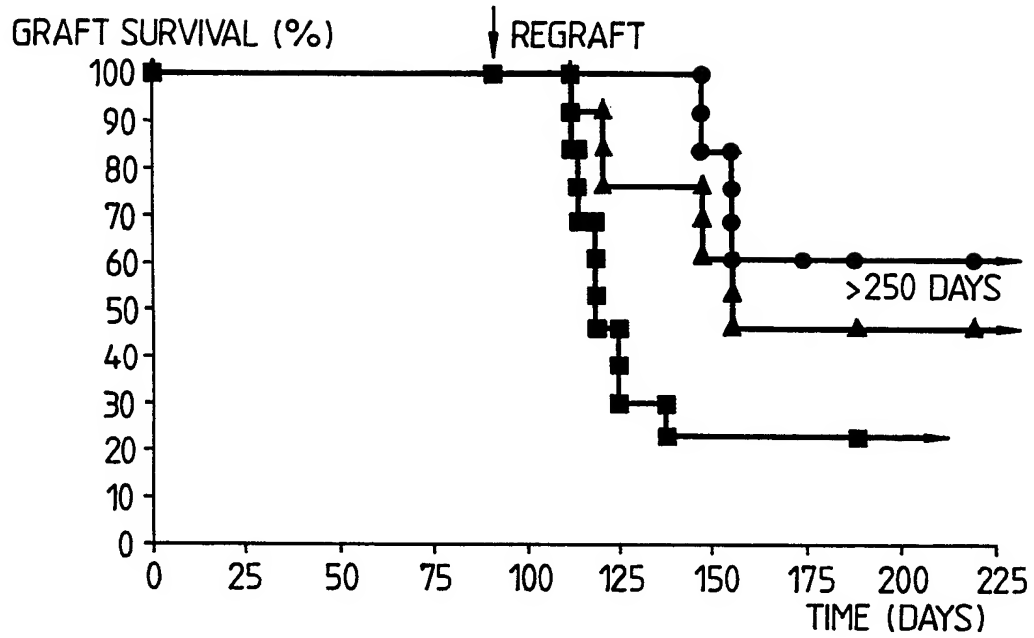
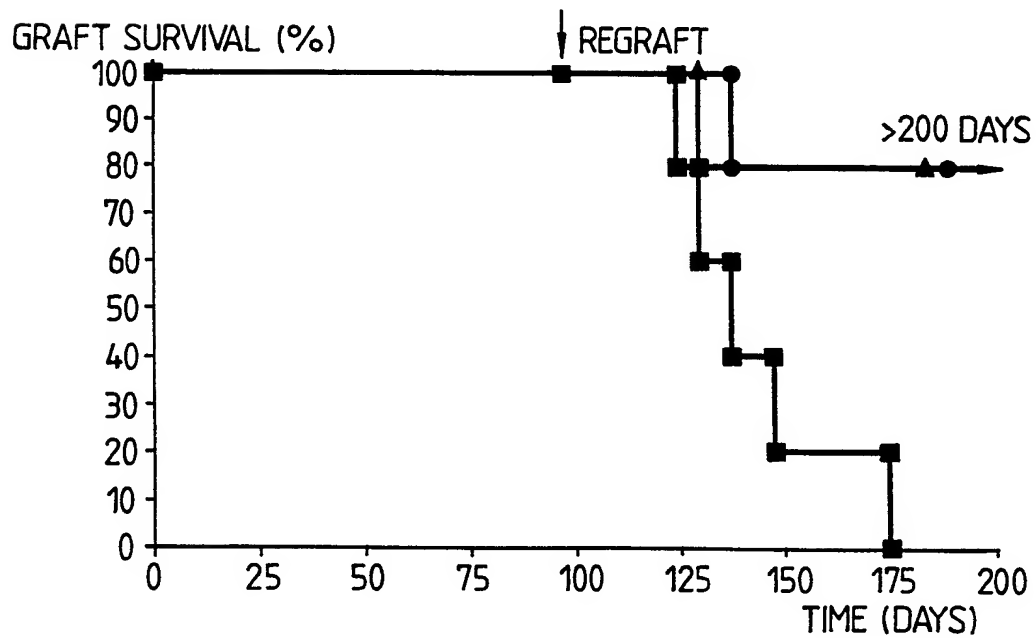
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Fig.8.*Fig.9.***SUBSTITUTE SHEET**

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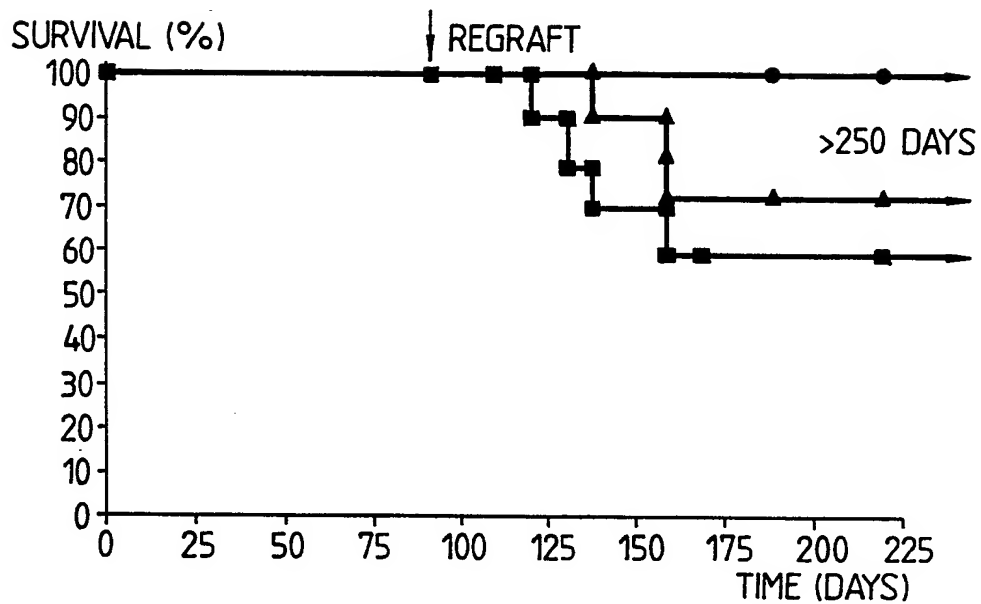
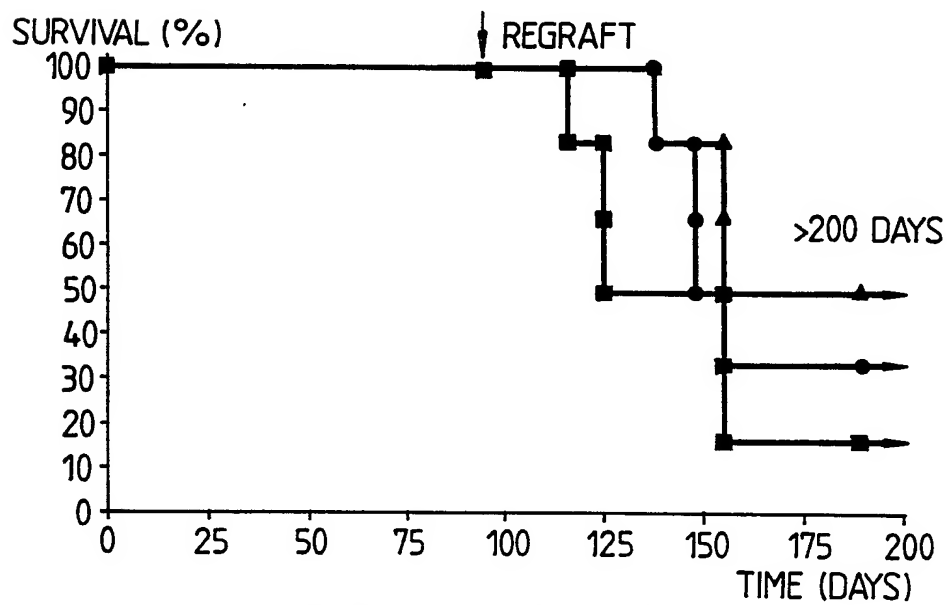
Fig.10a.*Fig.10b.***SUBSTITUTE SHEET**

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Fig.12a.*Fig.12b.*

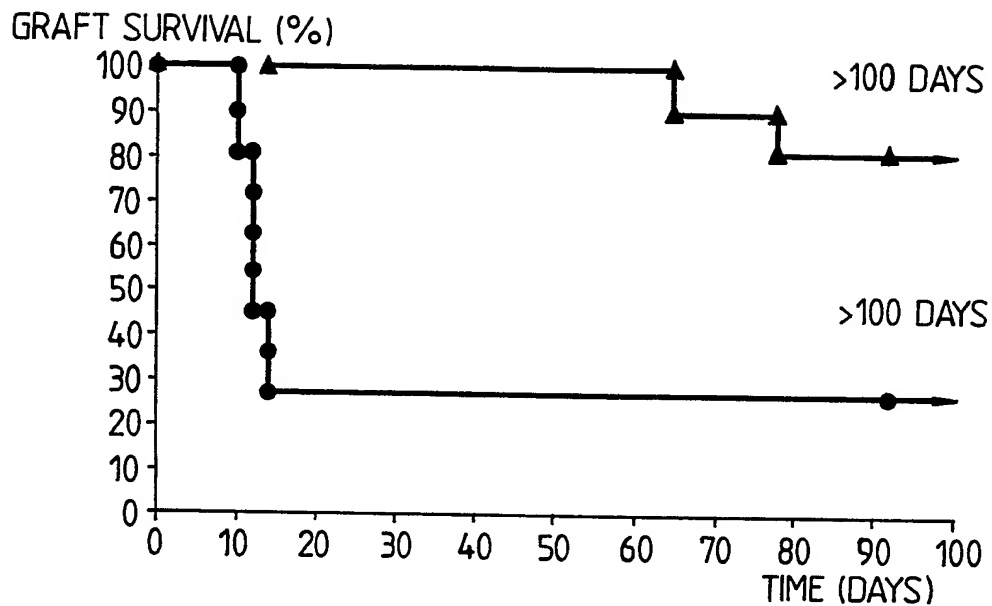
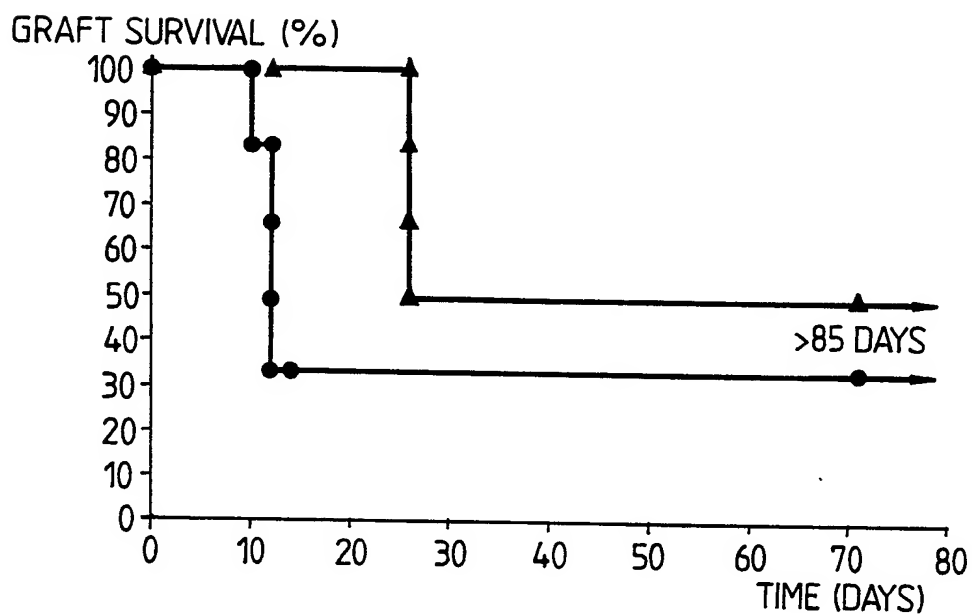
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Fig.12c.*Fig.12d.*

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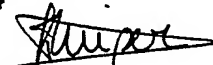
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Fig.13a.*Fig.13b.***SUBSTITUTE SHEET**

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 90/00840

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁵ : C 12 P 21/08, A 61 K 39/395		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁵	C 12 P, A 61 K, C 07 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Transplantation Proceedings, volume XIX, no. 5, October 1987, Grune & Stratton, Inc., (New York, US), M. Jonker et al.: "Effects of CD4 and CD8 specific monoclonal antibodies in vitro and in vivo an T cells and their relation to the allograft response in rhesus monkeys", pages 4308-4314 see the whole article --	1-14
X	Immunol. Cell Biol., volume 67, no. 1, January 1989, (Adelaide, AU), B. Charlton et al.: "Tolerance to aggregated human IgG but not ovalbumin can be induced by concurrent administration of depleting or non- depleting anti-L3T4 monoclonal anti- bodies", pages 1-7 see page 4, right-hand column, line 21 - page 6, right-hand column, line 4 -- ./.	3,5,6
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
21st September 1990		16 OCT 1990
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		Mme N. KUIPER 

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁾ with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	<p>The Journal of Immunology, volume 142, no. 5, 1 March 1989, The American Association of Immunologists, (Baltimore, US), N.L. Carteron et al.: "Treatment of murine lupus with F(ab')₂ fragments of monoclonal antibody to L3T4", pages 1470-1475 see the abstract</p> <p style="text-align: center;">--</p>	3,5,6
X	<p>Eur. J. Immunol., volume 17, 1987, VCH Verlagsgesellschaft mbH, (Weinheim, DE), S. Qin et al.: "CD4 monoclonal antibody pairs for immunosuppression and tolerance induction", pages 1159-1165 see page 1164, right-hand column, lines 15-29 cited in the application</p> <p style="text-align: center;">-----</p>	3,5,6

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers*), because they relate to subject matter not required to be searched by this Authority, namely:

*) Claims: 15-17 PCT Rule 39.1(iv): Methods for treatment of human or animal body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.